Method for the production of protamine

The present invention generally relates to the fields of
genetic engineering and biochemistry. In particular, it
relates to a novel method for the production of protamine as
well as to novel compositions containing protamine as active
ingredient. Furthermore, it relates to the use of said
compositions for killing or inhibiting microbial cells.

The immoderate use of different antibiotics during the last
six decades caused the emergence and the dissemination of
bacterial populations expressing resistance to these
substances. Interactions between microbial resistance and
antibacterial agents occur either directly by the development
of resistance to the agent used, to agents of the same class,
or in an indirect way by the selection of resistant organisms
when patients are treated with antibiotics, when the
environment is contaminated with antibiotics (hospital) or
when antibacterial agents are applied in agriculture and
animal husbandry. Antimicrobial selection as well as
contagion are the decisive processes responsible for the
global spread and linking of resistance genes.

There is increased concern that because of the widespread use
of antibiotics and the continuous increase of bacterial
resistance, the pharmaceutical industry may no longer be able
to develop effective novel antibiotics sufficiently rapidly
(JACK et al. 1995). This fact quickens interest in
microbially produced antibacterial peptides. In recent years,
more than fifty antibacterial peptides produced by lactic
acid bacteria have been isolated (NES and HOLO, 2000). These
so-called bacteriocins contain 20-60 amino acids, are

inhibitory in a nanomolar range and cause membrane
permeabilization and leakage of sensitive cells. They are
secreted by some bacterial strains, which are thus adapted to
compete against other microorganisms in the same
5   environmental compartment (NAVARRO et al. 2000).

Only a few microbially produced substances are known to be
effective against gram-positive as well as gram-negative
bacteria (BLACKBURN et al. 1989; STEVENS et al. 1991; KALCHAYANAND
10 et al. 1992). One promising substance of biogenic origin
possessing a broad antimicrobial spectrum (JOHANSEN et al.
1995; JOHANSEN et al. 1997; HANSEN and GILL 2000) is protamine,
a basic peptide (pI >10) consisting of 32 amino acids, of
which 21 are arginine (ANDO et al. 1973). Protamine is found
15 enriched in sperm, e.g. in salmon spermatozoan nuclei,
compacting DNA and taking the position of histones during the
maturation of the spermatids (LOUIE and DIXON, 1974). Its
effect on gram-negative bacteria is reported to be lower than
on gram-positive bacteria (ISLAM et al. 1984; MULHOLLAND and
20 MELLERSH 1987; YANAGIMOTO 1992; JOHANSEN et al. 1995).

There is an extremely high degree of sequence conservation in
the coding and 3´ untranslated regions of different rainbow
trout protamine genes (AIKEN et al. 1983), showing cross-
25 hybridization under less stringent conditions (SAKAI et al.
1981).

30

Protamine is also known for its antifungal activity (KAMAL et
al. 1986).

5   The antimicrobial effect of protamine is supposed to be
caused by its polycationic nature (HIRSCH 1958; JOHANSEN et al.
1995). A possible mode of action may consist in the binding
of the peptide to the outer membrane, causing a malfunction
and inducing channels into the membrane, as was proved for
10   other cationic peptides (CHRISTENSEN et al. 1988; KAGAN et al.
1990).

It has been demonstrated that protamine causes a disruption
of the cytoplasmatic membrane (JOHANSEN et al. 1997). It was
15   furthermore shown that polycationic peptides like protamine
can also activate autolysins, resulting in cell lysis, as
well as inhibit phosphorylase activity (JOHANSEN et al. 1996).
Protamine may also enter the cytoplasm, inhibiting genetic
transformation (ANTHOI and POPESCU 1979). Protamine penetrates
20   gram-negative bacteria and enhances the permeability of the
outer membrane (VAARA and VAARA 1983; VAARA 1992). Furthermore,
it deenergizes bacterial membranes and causes a reduction of
cellular ATP content (ASPEDON and GROISMAN, 1996) .

25   It has been shown that the antibacterial activity of
protamine is dependent on the amount of bivalent cations
stabilizing the outer membrane (JOHANSEN et al. 1997) and the
pH value (HANSEN and GILL 2000).

30   Protamine was shown to extend antibacterial effects against
food-spoiling bacteria (JOHANSEN et al. 1996). Interestingly,
STUMPE et al. (1998) found that OmpT, a protease from *E. coli*,

is able to degrade protamine before it enters the bacterial
cells. This fact has to be taken into consideration if
protamine is used as a disinfectant or a replacement for
antibiotics. It is of great importance that antibiotics are
5   no longer to be permitted in the food industry after the end
of 2003. Thus, new and effective substances have to be at
disposal before that time.

WO 96/06532 discloses a protamine composition for killing or
10 inhibiting microbial cells. The studies have been performed
with protamine from Salmon (P-4005) obtained from Sigma
Chemical Company (St. Louis, USA), dissolved in destilled
water, filter sterilized (0.2 μm) and used immediately after
preparation. Besides protamine or protamine sulphate, the
15 composition disclosed in WO 96/06532 additionally comprises a
cell-wall degrading enzyme and/or an oxidoreductase and
allegedly displays bactericidal, bacteriostatic, fungicidal
and/or fungistatic properties. Accordingly and due to its
broad range of activity against target organisms, protamine
20 containing preparations have already been suggested for
detergent and hard surface cleaning compositions and in
methods for killing microbial cells present on a hard
surface, for killing microbial cells or inhibiting growing
microbial cells present on laundry, for killing microbial
25 cells present on human or animal skin, mucous membranes,
wounds, bruises or in the eye, as well as for the
preservation of food, beverages, cosmetics, contact lens
products, food ingredients or enzyme compositions.

30 Furthermore, protamine and suitable derivatives thereof
display important physiological functions and have been
suggested for medical use. For example, protamine has proven

as a clinical heparin antagonist, e.g. to reduce post-operative bleeding, and is routinely administered after cardiac and vascular surgery to reverse the anticoagulant activity of heparin. In addition, protamine prolongs the adsorption of insulin, and is therefore combined with insulin to formulate protamine zinc insulin (PZI) and neutral protamine Hagedorn (NPH) insulin. Such formulations allow insulin-dependent diabetic patients to achieve euglycemia with less frequent insulin injections.

However, despite its universal use in clinical practice, current formulations of protamine are nevertheless toxic. Protamine toxicity ranges from mild hypotension to severe systemic vascular collapse requiring prompt intervention, or idiosyncratic fatal cardiac arrest. This drawback has been addressed in WO 00/55196 relating to low molecular weight (LMW) bioactive protamines and compositions, allegedly having reduced immunogenicity, antigenicity and/or toxicity compared to native protamine. According to the teaching of WO 00/55196, native salmine® (salmon) or clupeine® (herring) protamine commercially available from Sigma Chem. Co. is contacted with a proteolytic composition to generate LMW protamine fragments which can subsequently be used to select appropriate fragments, polypeptides, species of fractions of interest. However, WO 00/55196 does not provide any sequence information for the LMW protamine fragments.

It has thus been shown, that there is an increasing demand for protamine and/or its derivatives. Although it is stated in the art, that protamine can be isolated from mammals, amphibians and fish, and although is has long been speculated in the art to provide protamine as a recombinant protein, no

evidence of recombinant production has yet been published with the effect, that salmon (salmine®) and herring (clupeine®) protamine remain to be the only commercially available preparations to date.

5

Thus, the main object underlying the present invention is to overcome the drawbacks of the state of the art by providing a method for recombinantly producing bioactive protamine or functional fragments thereof enabling large-scale, cost-
10 effective and reliable production of this interesting substance.

Summary of the invention

The present invention provides means and methods of
15 transforming bacteria, yeast, fungi, insect, animal and plant cells, seeds, tissues and whole organisms in order to yield transformants capable of expressing a bioactive protamine polypeptide or functional fragment thereof (protamine). The present invention further provides means and methods to
20 recombinantly produce protamine using cells, tissues, organs or whole organisms which, after appropriate transformation, accumulate protamine. The present invention also provides DNA molecules encoding protamine derived from different sources and taxonomic groups of living organisms designed to be
25 suitable for carrying out the method of the invention, and plasmids or vector systems comprising said molecules. Furthermore, the present invention provides transgenic bacteria, yeast, fungi, insect, animal and plant cells, seeds, tissues and whole organisms that are capable of
30 producing protamine and contain the above DNA molecule(s) and/or that have been generated by use of the methods of the present invention. Additionally, the present invention

provides antibodies displaying a specific immunoreactivity with a protamine polypeptide which are suitable for diagnostic and screening purposes as well as for isolating and purifying said polypeptide. Furthermore, the present

5   invention provides a biological composition with bacteriocidal, bacteriostatic, fungicidal and/or fungistatic activity.

Additionally, the present invention provides pharmaceutical

10 preparations comprising a protamine polypeptide or functional active fragments thereof as active ingredient.

Finally, the present invention provides means and methods for use of the protamine and compositions according to the

15 invention.

20   Detailed description of the invention
In the present context, the term "bacteriocidal" is to be understood as capable of killing bacterial cells.

In the present context, the term "bacteriostatic" is to be

25 understood as capable of inhibiting bacterial growth, i.e. inhibiting growing bacterial cells.
In the present context, the term "fungicidal" is to be understood as capable of killing fungal cells.

30 In the present context, the term "fungistatic" is to be understood as capable of inhibiting fungal growth, i.e. inhibiting growing fungal cells.

The term "growing cell" is to be understood as a cell having access to a suitable nutrient and thus being capable of reproduction/propagation. By the term "non-growing cell" is meant a living, but dormant, cell, i.e. a cell in the non-growing, non-dividing, non-multiplying and non-energized state with metabolic processes at a minimum.

The term "microbial cells" denotes bacterial or fungal cells.

The terms "native", "of biological origin" and "from native or biological sources" are to be understood that a protamine polypeptide or functional fragment thereof is isolated, recovered or regenerated or that corresponding amino and nucleic acid sequence information is obtained or derived from biological material such as humans, animals or plants. These terms additionally include protamine polypeptides or functional fragments thereof which have been recombinantly produced according to the method of the invention on the basis of coding sequences obtained or derived from native or biological sources. Similarly, the term "of microbiological origin" denotes that the substance or compounds are recovered or regenerated from microbiological material such as bacteria, fungi, yeast or that a parent or native substance or compound is producible by a microbiological organism.

The terms "synthesized polypeptide" and "multimeric forms" refer to a synthesized assembly, i.e. a chain, built of peptide monomers. The protamine polypeptides and functional fragments thereof contemplated by the present invention are basic polypeptides, i.e. polylysins and polyarginins, and co-polymers thereof. It is preferred that the polypeptides have

a chain length of less than about 100 amino acids but is contemplated that polypeptides of less than ?? kD are useful. Preferably, the polypeptides to be produced or used or which are active ingredients in a composition or pharmaceutical

5 preparation according to the invention are of almost identical chain length or molecular weight but mixtures of polypeptides having various chain lengths or molecular weights are also useful.

10 Preferred basic proteins to be used in the compositions of the present invention are protamines, protamine sulphates, defensins, magainins, mellitin, cecropins and protegrins, with protamines and protamine sulphates being preferred.

15 The terms "protamine(s)", "protamine protein(s)" and "protamine (poly)peptide(s)" as used herein are meant to comprise any protamine amino acid sequence obtained or derived from native or biological sources including fragments thereof and multimeric forms of said amino acid sequence or

20 fragment thereof. Furthermore, these terms encompass (synthesized) polypeptides which are artificial and specifically designed for specific purposes and cannot be isolated from native or biological sources.

25 The term "biologically active" protamine denotes any protamine amino acid sequence displaying at least one biological activity of protamine as already known in the art. Included are also protamines which as such are not biologically active but which can be converted into

30 biologically active forms, e.g. by treating said protamines with substances such as chaperone proteins commercially available.

The term "functional fragment" of protamine denotes any protamine amino acid sequence or peptide retaining at least one of the biological activities as discussed in the art or

5   as mentioned above. Furthermore, said term comprises protamine amino acid sequences which retain at least one common structural or antigenic determinant as mentioned hereinbelow. In particular, functional fragments comprise those disclosed herein as well as those which can easily be

10   produced on the basis of information provided herein.

Although the method of recombinantly producing bioactive protamine has only been exemplified hereinbelow with respect to the use of DNA sequence information gathered from fish,

15   the present invention also provides DNA probes and sequence information which allow the person skilled in the art to clone the corresponding genes and/or cDNAs from other sources such as birds and mammals.

20   According to a preferred embodiment, these DNA sequences are in the form of cDNAs, genomic or manufactured (synthetic) DNA sequences and can be prepared as known in the art (see e.g. Sambrook et al., s.a.) or e.g. as specifically described hereinbelow.

25
     Given the guidance provided herein, the nucleic acids of the invention are obtainable according to methods well known in the art. For example, a DNA of the invention is obtainable by chemical synthesis, using polymerase chain reaction (PCR) or

30   by screening a genomic library or a suitable cDNA library prepared from a source believed to possess protamine and to express it at a detectable level.

Nucleic acid molecules and vectors according to the present
invention may be provided in a form isolated and/or purified
from their natural environment, in substantially pure or
homogeneous, or free or substantially free of nucleic acid

5   and or genes of the species of interest or origin other than
the relevant sequence. Nucleic acid according to the present
invention may include cDNA, RNA, genomic DNA and may be
wholly or partially synthetic. The term "isolate" where used
may encompass any of these possibilities.

10

Nucleic acid as herein provided or obtainable by use of the
disclosures herein, may be the subject of alteration by way
of one or more of addition, insertion, deletion or
substitution of nucleotides with or without altering the

15   encoded amino acid sequence (by virtue of the degeneracy of
the genetic code). Such altered forms of protamine
nucleotide sequences as herein provided or obtainable by use
of the disclosures herein can be easily and routinely tested
for both protamine function and protamine function in

20   accordance with standard techniques e.g. as provided herein.

Chemical methods for synthesis of a nucleic acid of interest

25   are known in the art and include triester, phosphite,
phosphoramidite and H-phosphonate methods, PCR and other
autoprimer methods as well as oligonucleotide synthesis on
solid supports. These methods may be used if the entire
nucleic acid sequence of the nucleic acid is known, or the

30   sequence of the nucleic acid complementary to the coding
strand is available. Alternatively, if the target amino acid
sequence is known, one may infer potential nucleic acid

sequences using known and preferred coding residues for each
amino acid residue.

An alternative means to isolate the gene encoding protamine
5   is to use PCR technology as described e.g. in section 14 of
Sambrook et al., 1989. This method requires the use of
oligonucleotide probes that will hybridise to protamine
nucleic acid. Strategies for selection of oligonucleotides
are described below.

10
Libraries are screened with probes or analytical tools
designed to identify the gene of interest or the protein
encoded by it. For cDNA expression libraries suitable means
include monoclonal or polyclonal antibodies that recognise
15   and specifically bind to protamine; oligonucleotides of about
20 to 80 bases in length that encode known or suspected
protamine cDNA from the same or different species; and/or
complementary or homologous cDNAs or fragments thereof that
encode the same or a hybridising gene. Appropriate probes for
20   screening genomic DNA libraries include, but are not limited
to oligonucleotides, cDNAs or fragments thereof that encode
the same or hybridising DNA; and/or homologous genomic DNAs
or fragments thereof.

25   A nucleic acid encoding protamine may be isolated by
screening suitable cDNA or genomic libraries under suitable
hybridisation conditions with a probe, i.e. a nucleic acid
disclosed herein including oligonucleotides derivable from
any of the sequences as set forth in SEQ ID NOS. 1, 3, 5, 7,
30   9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 28, 29, 30, 31, 32,
and 34. Suitable libraries are commercially available or can
be prepared e.g. from cell lines, tissue samples, and the
like.

As used herein, a probe is e.g. a single-stranded DNA or RNA that has a sequence of nucleotides that includes between 10 and 50, preferably between 15 and 30 and most preferably at

5   least about 20 contiguous bases that are the same as (or the complement of) an equivalent or greater number of contiguous bases, e.g. as set forth in any of the SEQ ID's mentioned above. The nucleic acid sequences selected as probes should be of sufficient length and sufficiently unambiguous so that

10 false positive results are minimised. The nucleotide sequences are usually based on conserved or highly homologous nucleotide sequences or regions of protamine as already mentioned hereinbefore. The nucleic acids used as probes may be degenerate at one or more positions. The use of degenerate

15 oligonucleotides may be of particular importance where a library is screened from a species in which preferential codon usage in that species is not known.

Preferred regions from which to construct probes include 5'
20 and/or 3' coding sequences, sequences predicted to encode ligand binding sites, and the like. For example, either the full-length cDNA clone disclosed herein or fragments thereof can be used as probes. Preferably, nucleic acid probes of the invention are labelled with suitable label means for ready

25 detection upon hybridisation. For example, a suitable label means is a radiolabel. The preferred method of labelling a DNA fragment is by incorporating α^{32P} dATP with the Klenow fragment of DNA polymerase in a random priming reaction, as is well known in the art. Oligonucleotides are usually end-

30 labelled with γ^{32P}-labelled ATP and polynucleotide kinase. However, other methods (e.g. non-radioactive) may also be used to label the fragment or oligonucleotide, including e.g.

enzyme labelling, fluorescent labelling with suitable
fluorophores and biotinylation.

After screening the library, e.g. with a portion of DNA
5   including substantially the entire protamine-encoding
sequence or a suitable oligonucleotide based on a portion of
said or equivalent DNA, positive clones are identified by
detecting a hybridisation signal; the identified clones are
characterised by restriction enzyme mapping and/or DNA
10   sequence analysis, and then examined, e.g. by comparison with
the sequences set forth herein, to ascertain whether they
include DNA encoding a complete protamine (i.e., if they
include translation initiation and termination codons). If
the selected clones are incomplete, they may be used to
15   rescreen the same or a different library to obtain
overlapping clones. If the library is genomic, then the
overlapping clones may include exons and introns. If the
library is a cDNA library, then the overlapping clones will
include an open reading frame. In both instances, complete
20   clones may be identified by comparison with the DNAs and
deduced amino acid sequences provided herein.

In order to detect any abnormality of endogenous protamine,
genetic screening may be carried out using the nucleotide
25   sequences of the invention as hybridisation probes.

It is envisaged that the nucleic acids of the invention can
be readily modified by nucleotide substitution, nucleotide
deletion, nucleotide insertion or inversion of a nucleotide
30   stretch, and any combination thereof. Such mutants can be
used e.g. to produce a protamine mutant that has an amino
acid sequence differing from the protamine sequences as found

in nature. Mutagenesis may be predetermined (site-specific) or random. A mutation which is not a silent mutation must not place sequences out of reading frames and preferably will not create complementary regions that could hybridise to produce

5   secondary mRNA structure such as loops or hairpins.

Furthermore, the present invention envisages and enables the use of the sequence data provided herein to conduct relational and functional genomic studies. Relational studies

10 are used as adjuncts to sequencing and mapping activities, and are designed to provide interesting, and potentially important, hints about biological function including e.g. homology searches, secondary structure correlations, differential cDNA screening, expression cloning, genetic

15 linkage analysis, positional cloning and mutational analysis. In contrast to relational studies, functional studies generally make use of cells or animals to attempt a more direct correlation of sequence and biological function and include e.g. screening for phenotypic changes in systems such

20 as yeast, flies, mitochondria, human tissues, mice, and frogs, using gene "knockouts" or other methods intended to control gene expression or protein action in order to provide information useful in relating sequences to function. These techniques as such are well-known in the art.

25

Use of the above approaches should preferably achieve one or more of the following criteria: (a) inhibition of the gene sequence should be sequence-specific in order to substantially eliminate false-positive results; (b) should

30 have a broad based applicability, i.e. it should be possible to work with both high and low abundance genes, as well as with sequences whose product may be intracellular, membrane-

associated, or extracellular; (c) should be applicable in models predictive of the (human) condition of interest; (d) should allow dose-response studies to be conducted e.g. in order to determine the dose at which the target is most

5   affected; (e) the amount of information needed for target validation studies preferably should be minimal, i.e. the technique e.g. allows for dealing directly with ESTs without the former requirement of obtaining full-length gene sequences, promoter and other regulatory information, or

10   protein sequence/structure; (f) should be useable in a high-throughput mode.

Accordingly, the present invention provides sufficient guidance to apply all approaches and techniques described

15   above including "knockouts", intracellular antibodies, aptamers, antisense oligonucleotides, and ribozymes. In a preferred embodiment of the present invention, protamine-specific antisense oligonucleotides derived from any of the sequences as set forth in SEQ ID NOS. 1, 3, 5, 7, 9, 11, 13,

20   15, 17, 19, 21, 23, 25, 27, 28, 29, 30, 31, 32, and 34 can be used in order to identify the minimum sequence information needed to generate bioactive protamine. In a further preferred embodiment, use is made of specifically designed ribozymes which deliver optimized sequence-specific

25   inhibition by manipulating elements inherent to their mechanism of action. For example, ribozymes can be designed to bind only to their targets, and by chosing a target sequence of 15 nucleotides - well within the informational limits of typical ESRs - there is assurance, on a statistical

30   basis, that the target sequence will appear only once in the genome. Accordingly, the invention generally provides ribozymes specifically designed to interact only with its

target which is expected to appear only once in the genome, ensuring a high degree of assurance that only the specific target has been inhibited. More particularly, the invention provides ribozymes which are uniquely equipped to deliver several types of important controls that can verify that inhibition of a specific mRNA target was the actual cause of alteration of protamine-mediated conditions or phenotypes. It is known, for example, that mutating the ribozyme's catalytic core renders it incapable of cleavage but still functional in terms of highly specific binding to its target. These "inactivated" ribozymes produce either no or substantially reduced target inhibition relative to the active ribozyme – making them a very effective negative control. Alternatively, the catalytic core can be maintained in its active form, but the target arms are modified such that they will not bind the target sequence. If nonspecific cleavage is occurring, such a construct should show activity. Since ribozymes contain noncontiguous binding arms, each of the ribozyme's two binding arms binds seperately and adds to ribozyme selectivity while maintaining specificity. Due to the low binding strength of such noncontiguous binding arms compared to e.g. contiguous antisense binding, any mismatches between the ribozyme and the target sequence will not be expected to bind effectively and thus allow the target to fall off before cleavage.

For the approaches and techniques as exemplified above, both the entire sequence as set forth in any of SEQ ID NOS. 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 28, 29, 30, 31, 32, and 34, as well as fragments thereof, in particular those described herein or those which encode an amino acid sequence selected from the group consisting of amino acid sequences

extending from position 9 to position 18, from position 9 to position 20, from position 9 to position 21, from position 9 to position 22, from position 9 to position 23, from position 10 to position 20, from position 10 to position 21, from position 10 to position 22, from position 10 to position 23, and from position 10 to 24 of SEQ ID NO. 2 , can be used.

If required, nucleic acids encoding protamine-related proteins or polypeptides can be cloned from cells or tissues according to established procedures using probes derived from protamine. In particular, such DNAs can be prepared by:

a) isolating mRNA from suitable cells or tisues, selecting the desired mRNA, for example by hybridisation with a DNA probe or by expression in a suitable expression system, and screening for expression of the desired polypeptide, preparing single-stranded cDNA complementary to that mRNA, then double-stranded cDNA therefrom, or

b) isolating cDNA from a cDNA library and selecting the desired cDNA, for example using a DNA probe or using a suitable expression system and screening for expression of the desired polypeptide, or

c) incorporating the double-stranded DNA of step a) or b) into an appropriate expression vector,

d) transforming appropriate host cells with the vector and isolating the desired DNA.

Polyadenylated messenger RNA (step a) is isolated by known methods. Isolation methods involve, for example, homogenizing cells in the presence of a detergent and a ribonuclease inhibitor, for example heparin, guanidinium isothiocyanate or

mercaptoethanol, extracting the mRNA with a chloroform-phenol mixture, optionally in the presence of salt and buffer solutions, detergents and/or cation chelating agents, and precipitating mRNA from the remaining aqueous, salt-
5   containing phase with ethanol, isopropanol or the like. The isolated mRNA may be further purified by centrifuging in a caesium chloride gradient followed by ethanol precipitation and/or by chromatographic methods, for example affinity chromatography, for example chromatography on oligo(dT)
10 cellulose or on oligo(U) sepharose. Preferably, such purified total mRNA is fractionated according to size by gradient centrifugation, for example in a linear sucrose gradient, or chromatography on suitable size fractionation columns, for example on agarose gels.

15

The desired mRNA is selected by screening the mRNA directly with a DNA probe, or by translation in suitable cells or cell-free systems and screening the obtained polypeptides. The selection of the desired mRNA is preferably achieved
20 using a DNA hybridisation probe, thereby avoiding the additional step of translation. Suitable DNA probes are DNAs of known nucleotide sequence consisting of at least 17 nucleotides derived from DNAs encoding protamine or a related protein. Alternatively, EST sequence information can be used
25 to generate suitable DNA probes.

Synthetic DNA probes are synthesised according to known methods as detailed hereinbelow, preferably by stepwise condensation using the solid phase phosphotriester, phosphite
30 triester or phosphoramidite method, for example the condensation of dinucleotide coupling units by the phosphotriester method. These methods are adapted to the

synthesis of mixtures of the desired oligonucleotides by using mixtures of two, three or four nucleotides dA, dC, dG and/or dT in protected form or the corresponding dinucleotide coupling units in the appropriate condensation step as
5   described by Y. Ike et al. (Nucleic Acids Research 11, 477, 1983).

For hybridisation, the DNA probes are labelled, for example radioactively labelled by the well known kinase reaction. The
10 hybridisation of the size-fractionated mRNA with the DNA probes containing a label is performed according to known procedures, i.e. in buffer and salt solutions containing adjuncts, for example calcium chelators, viscosity regulating compounds, proteins, irrelevant DNA and the like, at
15 temperatures favouring selective hybridisation, for example between 0°C and 80°C, for example between 25°C and 50°C or around 65°C, preferably at around 20° lower than the hybrid double-stranded DNA melting temperature.

20 Fractionated mRNA may be translated in cells, for example frog oocytes, or in cell-free systems, for example in reticulocyte lysates or wheat germ extracts. The obtained polypeptides are screened for protamine activity or for reaction with antibodies raised against protamine or the
25 protamine-related protein, for example in an immunoassay, for example radioimmunoassay, enzyme immunoassay or immunoassay with fluorescent markers. Such immunoassays and the preparation of polyclonal and monoclonal antibodies are well known in the art and are applied accordingly. According to
30 the invention there are provided polyclonal antibodies.

The preparation of a single-stranded complementary DNA (cDNA)
from the selected mRNA template is well known in the art, as
is the preparation of a double-stranded DNA from a single-
stranded DNA. The mRNA template is incubated with a mixture
5   of deoxynucleoside triphosphates, optionally radioactively
labelled deoxynucleoside triphosphates (in order to be able
to screen the result of the reaction), a primer sequence such
as an oligo-dT residue hybridising with the poly(A) tail of
the mRNA and a suitable enzyme such as a reverse
10 transcriptase for example from avian myeloblastosis virus
(AMV). After degradation of the template mRNA for example by
alkaline hydrolysis, the cDNA is incubated with a mixture of
deoxynucleoside triphosphates and a suitable enzyme to give a
double-stranded DNA. Suitable enzymes are for instance a
15 reverse transcriptase, the Klenow fragment of E. coli DNA
polymerase I or T4 DNA polymerase. Usually, a hairpin loop
structure formed spontaneously by the single-stranded cDNA
acts as a primer for the synthesis of the second strand. This
hairpin structure is removed by digestion with S1 nuclease.
20 Alternatively, the 3'-end of the single-stranded DNA is first
extended by homopolymeric deoxynucleotide tails prior to the
hydrolysis of the mRNA template and the subsequent synthesis
of the second cDNA strand.

25 In the alternative, double-stranded cDNA is isolated from a
cDNA library and screened for the desired cDNA (step b). The
cDNA library is constructed by isolating mRNA from suitable
cells, and preparing single-stranded and double-stranded cDNA
therefrom as described above. This cDNA is digested with
30 suitable restriction endonucleases and incorporated into λ
phage, for example λ charon 4A or λ gt11 following
established procedures. The cDNA library replicated on

nitrocellulose membranes is screened by using a DNA probe as
described hereinbefore, or expressed in a suitable expression
system and the obtained polypeptides screened for reaction
with an antibody specific for the desired protamine.

5

A variety of methods are known in the art for the
incorporation of double-stranded cDNA into an appropriate
vector (step c). For example, complementary homopolymer
tracts may be added to the double-stranded DNA and the vector

10 DNA by incubation in the presence of the corresponding
deoxynucleoside triphosphates and an enzyme such as terminal
deoxynucleotidyl transferase. The vector and double-stranded
DNA are then joined by base pairing between the complementary
homopolymeric tails and finally ligated by specific joining

15 enzymes such as ligases. Other possibilities are the addition
of synthetic linkers to the termini of the double-stranded
DNA, or the incorporation of the double-stranded DNA into the
vector by blunt- or staggered-end ligation.

20 The transformation of appropriate host cells with the
obtained hybrid vector (step d) and the selection of
transformed host cells (step e) are well known in the art.
Hybrid vectors and host cells may be particularly suitable
for the production of DNA, or for the production of the

25 desired protamine.

In addition to being useful for the production of recombinant
protamine protein, these nucleic acids are also useful as
probes, thus readily enabling those skilled in the art to

30 identify and/or isolate nucleic acid encoding protamine. The
nucleic acid may be unlabelled or labelled with a detectable
moiety. Furthermore, the nucleic acids according to the
invention are useful e.g. in a method determining the

presence or even quantity of protamine specific nucleic acid, said method comprising hybridising the DNA (or RNA) encoding (or complementary to) protamine to test sample nucleic acid and determining the presence and, optionally, the amount of protamine. In another aspect, the invention provides a nucleic acid sequence that is complementary to, or hybridises under stringent conditions to, a nucleic acid sequence encoding protamine. These oligonucleotides can efficiently be used in antisense and/or ribozyme approaches, including gene therapy.

The invention also provides a method for amplifying a nucleic acid test sample comprising priming a nucleic acid polymerase (chain) reaction with nucleic acid (DNA or RNA) encoding (or complementary to) protamine.

The DNA-sequences of the present invention can thus be used as a guideline to define new PCR primers for the cloning of substantially homologous DNA sequences from other sources. In addition they and such homologous DNA sequences can be integrated into vectors by methods known in the art and described by e.g. Sambrook et al. (s.a.) to express or overexpress the encoded polypeptide(s) in appropriate host systems. However, a person skilled in the art knows that also the DNA-sequences themselves can be used to transform the suitable host systems of the invention to get overexpression of the encoded polypeptide or functional fragment thereof.

The nucleic acid molecule may be in the form of a recombinant and preferably replicable vector for example a plasmid, cosmid, phage or binary vector, e.g. suitable for use with bacteria such as those belonging to the genus of Rhodopseudomonas, Pseudomonas and Escherichia. The nucleic

acid may be under the control of an appropriate promoter and regulatory elements for expression in a host cell such as a microbial, e.g. bacterial, animal (mammalian), yeast or plant cell. In the case of genomic DNA, this may contain its own

5   promoter and regulatory elements and in the case of cDNA this may be under the control of an appropriate promoter and regulatory elements for expression in the host cell. Thus, the nucleotide sequence of SEQ ID NO. 32 (for example) may be placed under the control of a heterologous promoter.

10   Similarly, a protamine homologue sequence from another species may be operably linked to a promoter other than that with which it is naturally associated. However, a vector including nucleic acid according to the present invention need not include a promoter, particularly if the vector is to

15   be used to introduce the nucleic acid into cells for recombination into the genome or when used for promoter-trapping.

Those skilled in the art are well able to construct vectors

20   and design protocols for recombinant gene expression. Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including promoter sequences, terminator fragments, polyadenylation sequences, enhancer sequences, marker genes and other sequences as

25   appropriate. For further details see, for example, Molecular Cloning: a Laboratory Manual: 2nd edition, Sambrook et al, 1989, Cold Spring Harbor Laboratory Press. Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs,

30   mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in Current Protocols in Molecular Biology, Second

Edition, Ausubel et al. eds., John Wiley & Sons, 1992. The
disclosures of Sambrook et al. and Ausubel et al. are
incorporated herein by reference. Specific procedures and
vectors previously used with wide success upon plants are

5   described by Bevan (Nucl. Acids Res. 12, 8711-8721 (1984))
and Guerineau and Mullineaux (1993) (Plant transformation and
expression vectors. In: Plant Molecular Biology Labfax (Croy
RRD ed) Oxford, BIOS Scientific Publishers, pp 121-148).

10 As outlined above, the present invention thus provides
specific DNA molecules as well as plasmid or vector systems
comprising the same which comprise a DNA sequence within an
operable expression cassette capable of directing production
of a functionally active protamine. Preferably, said DNA

15 molecules further comprise at least one selectable marker
gene or cDNA operably linked to a constitutive, inducible or
tissue-specific promoter sequence allowing its expression in
bacteria, yeast, fungi, insect, animal or plant cells, seeds,
tissues or whole organisms. If plastid-containing material is

20 selected for transformation it is preferred that the coding
nucleotide sequence is fused with a suitable plastid transit
peptide encoding sequence, both of which preferably are
expressed under the control of a tissue-specific or
constitutive promoter.

25

The nucleic acid as provided by the present invention may be
placed under the control of an inducible gene promoter thus
placing expression under the control of the user.

30 In a further aspect the present invention provides a gene
construct including an inducible promoter operatively linked
to a nucleotide sequence provided by the present invention.
As discussed, this enables control of expression of the gene.

The invention also provides suitable hosts or host cells transformed with said gene construct and methods including introduction of such a construct into a suitable host or host cell and/or induction of expression of a construct within a

5   suitable host or host cell, e.g by application of a suitable stimulus, such as an effective exogenous inducer.

The term "inducible" as applied to a promoter is well understood by those skilled in the art. In essence,

10 expression under the control of an inducible promoter is "switched on" or increased in response to an applied stimulus (which may be generated within a cell or provided exogenously). The nature of the stimulus varies between promoters. Some inducible promoters cause little or

15 undetectable levels of expression (or no expression) in the absence of the appropriate stimulus. Other inducible promoters cause detectable constitutive expression in the absence of the stimulus. Whatever the level of expression is in the absence of the stimulus, expression from any inducible

20 promoter is increased in the presence of the correct stimulus. The preferable situation is where the level of expression increases upon application of the relevant stimulus by an amount effective to alter a phenotypic characteristic. Thus an inducible (or "switchable") promoter

25 may be used which causes a basic level of expression in the absence of the stimulus which level is too low to bring about a desired phenotype (and may in fact be zero). Upon application of the stimulus, expression is increased (or switched on) to a level which brings about the desired

30 phenotype. One example of an inducible promoter is the ethanol inducible gene switch disclosed in Caddick et al (1998) Nature Biotechnology 16: 177-180. Many other examples will be known to those skilled in the art.

An example of a suitable plant promoter may be the Cauliflower Mosaic Virus 35S (CaMV 35S) gene promoter that is expressed at a high level in virtually all plant tissues
5   (Benfey et al, (1990) EMBO J 9: 1677-1684).

Polypeptides according to the invention include protamine and derivatives thereof which retain at least one common structural determinant of protamine.

10
"Common structural determinant" means that the derivative in question possesses at least one structural feature of protamine. Structural features includes possession of an epitope or antigenic site that is capable of cross-reacting
15   with antibodies raised against a naturally occurring or denatured protamine polypeptide or fragment thereof, possession of amino acid sequence identity with protamine and features having common a structure/function relationship. Thus, protamine as provided by the present invention includes
20   splice variants encoded by mRNA generated by alternative splicing of a primary transcript, amino acid mutants, glycosylation variants and other covalent derivatives of protamine which retain the physiological and/or physical properties of protamine. Exemplary derivatives include
25   molecules wherein the protein of the invention is covalently modified by substitution, chemical, enzymatic, or other appropriate means with a moiety other than a naturally occurring amino acid. Such a moiety may be a detectable moiety such as an enzyme or a radioisotope. Further included
30   are naturally occurring variants or homologues of protamine found with a particular species. Such a variant or homologue may be encoded by a related gene of the same gene family, by

an allelic variant of a particular gene, or represent an
alternative splicing variant of the protamine gene.

The protamine homologue may at the nucleotide level have
5   homology with a nucleotide sequence as set forth in any of
SEQ ID NOS. 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25,
27, 28, 29, 30, 31, 32, and 34, or may encode a polypeptide
which has homology with the protamine polypeptides or
functional fragments according to the invention as set forth
10 in any of SEQ ID NOS. 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22,
24, and 34, preferably at least about 50%, 51%, 52%, 53%, 54%
or 55%, or at least about 60%, or at least about 65%, or at
least about 70%, or at least about 75%, or at least about 80%
homology, or at least about 90% homology. Most preferably,
15 at least about 95% or greater homology. (Determination of
homology at the amino acid level is discussed further below.)

In certain embodiments, a polypeptide allele, variant,
derivative, mutant derivative, mutant or homologue of the
20 specific sequences may show little overall homology, say
about 20%, or about 25%, or about 30%, or about 35%, or about
40% or about 45%, with the protamine amino acid sequences
such as those set forth in any of SEQ ID NOS. 2, 4, 6, 8, 10,
12, 14, 16, 18, 20, 22, 24, 26, and 33, including fragments
25 extending from position 9 to position 18, from position 9 to
position 20, from position 9 to position 21, from position 9
to position 22, from position 9 to position 23, from position
10 to position 20, from position 10 to position 21, from
position 10 to position 22, from position 10 to position 23,
30 and from position 10 to 24 of SEQ ID NO. 2. Preferably, any
such polypeptide alleles, variants, derivatives, mutant
derivatives, mutants or homologues have 95%, 96%, 97%, 98%,
99%, or 100% identity with any of the sequences mntioned

above. However, in any functionally significant domains or regions the amino acid percentage identity may be much higher, for example, as discussed above for the functional fragments disclosed herein. Putative functionally significant

5   domains or regions can be identified using processes of bioinformatics, including comparison of the sequences of homologues.

Functionally significant domains or regions of different

10   polypeptides may be combined for expression from encoding nucleic acid as a fusion protein. For example, particularly advantageous or desirable properties of different homologues may be combined in a hybrid protein, such that the resultant expression product, with protamine function, may include

15   fragments of various parent proteins.

Similarity of amino acid sequences may be as defined and determined by the TBLASTN program, of Altschul et al. (1990) J. Mol. Biol. 215: 403-10, which is in standard use in the

20   art.   In particular, TBLASTN 2.0 may be used with Matrix BLOSUM62 and GAP penalties: existence: 11, extension: 1. Another standard program that may be used is BestFit, which is part of the Wisconsin Package, Version 8, September 1994, (Genetics Computer Group, 575 Science Drive, Madison,

25   Wisconsin, USA, Wisconsin 53711). BestFit makes an optimal alignment of the best segment of similarity between two sequences. Optimal alignments are found by inserting gaps to maximize the number of matches using the local homology algorithm of Smith and Waterman (Adv. Appl. Math. (1981) 2:

30   482-489). Other algorithms include GAP, which uses the Needleman and Wunsch algorithm to align two complete sequences that maximizes the number of matches and minimizes

the number of gaps. As with any algorithm, generally the default parameters are used, which for GAP are a gap creation penalty = 12 and gap extension penalty = 4. Alternatively, a gap creation penalty of 3 and gap extension penalty of 0.1

5   may be used. The algorithm FASTA (which uses the method of Pearson and Lipman (1988) *PNAS USA* **85**: 2444-2448) is a further alternative.

Use of either of the terms "homology" and "homologous" herein
10   does not imply any necessary evolutionary relationship between compared sequences, in keeping for example with standard use of terms such as "homologous recombination" which merely requires that two nucleotide sequences are sufficiently similar to recombine under the appropriate
15   conditions. Further discussion of polypeptides according to the present invention, which may be encoded by nucleic acid according to the present invention, is found herein.

Protamine fragments that are functional in the context of the
20   present invention are also included within the ambit of the invention. Examples for such functional fragments include amino acid sequences extending from position 9 to position 18, from position 9 to position 20, from position 9 to position 21, from position 9 to position 22, from position 9
25   to position 23, from position 10 to position 20, from position 10 to position 21, from position 10 to position 22, from position 10 to position 23, and from position 10 to 24 of SEQ ID NO. 2.

30   The obtaining of homologues is later discussed herein, but briefly here it should be pointed out that the nucleotide sequence information provided herein, or any part thereof, may be used in a data-base search to find homologous

sequences, expression products of which can be tested for
protamine function.

By sequencing homologues, studying their expression patterns

5   and examining the effect of altering their expression, genes
carrying out a similar function to protamine are obtainable.
Of course, mutants, variants and alleles of these sequences
are included within the scope of the present invention in the
same terms as discussed above for the protamine genes

10 discussed herein, although it should be noted that homologue
sequences pre-existing on databases, such as any identified
herein, may be excluded from one or more aspects or
embodiments of the present invention while included in one or
more other aspects.

15
Derivatives which retain common structural features can be
fragments of protamine such as those disclosed herein.
Fragments of protamine comprise individual domains thereof,
as well as smaller polypeptides derived from the domains.

20 Preferably, smaller polypeptides derived from protamine
according to the invention define a single feature which is
characteristic of protamine. Fragments may in theory be
almost any size, as long as they retain one feature of
protamine. Preferably, fragments will be between 5 and 20

25 amino acids in length. Longer fragments are regarded as
truncations of the full-length protamine and generally
encompassed by the term "protamine". Exemplary fragments of a
protamine polypeptide include amino acid sequences extending
from position 9 to position 18, from position 9 to position

30 20, from position 9 to position 21, from position 9 to
position 22, from position 9 to position 23, from position 10
to position 20, from position 10 to position 21, from

position 10 to position 22, from position 10 to position 23, and from position 10 to 24 of SEQ ID NO. 2.

5   Derivatives of protamine also comprise mutants thereof, which may contain amino acid deletions, additions or substitutions, subject to the requirement to maintain at least one feature characteristic of protamine. Thus, conservative amino acid substitutions may be made substantially without altering the nature of protamine, as may truncations from the 5' or 3'

10   ends. Deletions and substitutions may moreover be made to the fragments of protamine comprised by the invention. Protamine mutants may be produced from a DNA encoding protamine which has been subjected to *in vitro* mutagenesis resulting e.g. in an addition, exchange and/or deletion of one or more amino

15   acids. For example, substitutional, deletional or insertional variants of protamine can be prepared by recombinant methods and screened for immuno-crossreactivity with the native forms of protamine. Derivatives of protamine further comprise multimeric forms of protamine or functional fragments

20   thereof.

The present invention also provides polypeptides and derivatives of protamine which retain at least one common antigenic determinant of protamine.

25
"Common antigenic determinant" means that the derivative in question possesses at least one antigenic function of protamine. Antigenic functions includes possession of an epitope or antigenic site that is capable of cross-reacting

30   with antibodies raised against a naturally occurring or denatured protamine polypeptide or fragment thereof.

Derivatives which retain common antigenic determinants can be fragments of protamine. Fragments of protamine comprise individual domains thereof, as well as smaller polypeptides derived from the domains. Preferably, smaller polypeptides

5   derived from protamine according to the invention define a single epitope which is characteristic of protamine. Fragments may in theory be almost any size, as long as they retain one characteristic of protamine. Preferably, fragments will be between 5 and 20 amino acids in length. Longer

10   fragments are regarded as truncations of the full-length protamine and generally encompassed by the term "protamine". Derivatives of protamine further comprise multimeric forms of protamine or functional fragments thereof.

15   The present invention provides methods for recombinantly producing a protamine polypeptide comprising the steps of (a) expressing a polypeptide encoded by a DNA as outlined above in a suitable host, and (b) isolating said protamine polypeptide according to conventional techniques well known

20   in the art. In addition, there is provided a protein which is obtained or obtainable by use of the aforementioned process.

Preferably, the protein or derivative thereof of the invention is provided in isolated form. "Isolated" means that

25   the protein or derivative has been identified and is free of one or more components of its natural environment. Isolated protamine includes protamine in a recombinant cell culture. Protamine present in an organism expressing a recombinant protamine gene, whether the protamine protein is "isolated"

30   or otherwise, is included within the scope of the present invention.

The host material selected for transformation should express the gene(s) introduced, and is preferably homozygous for expression thereof. Generally, the gene will be operably linked to a promoter functionally active in the targeted host

5   cells of the particular plant, insect, animal or microorganism (such as e.g. fungi including yeast and bacteria). The expression should be at a level such that the characteristic desired from the gene is obtained. For example, the expression of a selectable marker gene should

10 provide for an appropriate selection of transformants yielded according to the methods of the present invention. Similarly, the expression of a gene coding for an enzyme displaying the desired activity of protamine should result in a transformant having a relatively higher content of the encoded gene

15 product as compared to that of the same species which is not subjected to the transformation method according to the present invention. On the other hand, it will generally be desired to limit the excessive expression of the gene of interest in order to avoid significantly adversely affecting

20 the normal physiology of the plant, insect, fungal, animal or microorganism, i.e. to the extent that cultivation thereof becomes difficult.

The gene encoding protamine can be used in expression

25 cassettes for expression in the transformed procaryotic or eucaryotic host cell, seed, tisue or whole organism. To achieve the objects of the present invention, i.e., to produce protamine in a target host of interest, the transformation is preferably carried out by use of an

30 operable expression cassette comprising a transcriptional initiation region linked to the gene encoding protamine.

The transcriptional initiation may be native or analogous to
the host or foreign or heterologous to the host. By foreign
is intended that the transcriptional initiation region is not
found in the wild-type host into which the transcriptional

5   initiation region is introduced.

In plant material, those transcriptional initiation regions
are of particular interest which are associated with storage
proteins, such as glutelin, patatin, napin, cruciferin, β-

10   conglycinin, phaseolin, or the like.

The transcriptional cassette will include, in 5' - 3' direc-
tion of transcription, a transcriptional and translational
initiation region, a DNA sequence encoding protamine or a

15   functional fragment thereof retaining its specific
immunogenic or biological activity, and a transcriptional and
translational termination region functional in the targeted
host material such as, e.g., plants or microorganims,
respectively. The termination region may be native with the

20   transcriptional initiation region, may be native with the DNA
sequence of interest, or may be derived from other sources.
Convenient termination regions suitable for plant material
are available from the Ti-plasmid of *A. tumefaciens* such as
the octopine synthase and nopaline synthase termination

25   regions [see also, Guerineau et al., (1991) *Mol. Gen. Genet.*
262, 141-144; Proudfoot, (1991) *Cell 64*, 671-674; Sanfacon et
al., (1991) *Gened Dev.* 5, 141-149; Mogen et al., (1990) *Plant
Cell 2*, 1261-1272; Munroe et al., (1990) *Gene 91*, 151-158;
Ballas et al., (1989), *Nucl. Acids Res. 17*, 7891-7903; Joshi

30   et al., (1987) *Nucl. Acids Res. 15*, 9627-9639].

For the expression of protamine in plant or plastid-
containing material, the coding sequence is preferably fused

to a sequence encoding a transit peptide which after expression and translation directs the translocation of the protein upon cleavage of the transit peptide to (plant) plastids, such as chloroplasts. For example, the protamine cDNA can be translationally fused to a sequence encoding for the transit peptide of the small subunit of ribulose-1,5-bis-phosphate carboxylase (rubisco) or to sequences coding for transit peptides of other plastid proteins. Such transit peptides are known in the art [see, for example, Von Heijne et al., (1991) *Plant Mol. Biol. Rep. 9*, 104-126; Clark et al., (1989) *J. Biol. Chem. 264*, 17544-17550; Della-Cioppa et al., (1987) *Plant Physiol. 84*, 965-968; Romer et al., (1993) *Biochim Biophys. Res. Commun. 196*, 1414-1421; and, Shah et al., (1986) *Science 233*, 478-481]. Any genes useful for carrying out the present invention can utilize native or heterologous transit peptides.

The construct can also include any other necessary regulators such as plant translational consensus sequences (Joshi, 1987, s.a.), introns [Luehrsen and Walbot, (1991) *Mol. Gen. Genet. 225*, 81-93] and the like, operably linked to the nucleotide sequence encoding protamine. Intron sequences within the coding gene desired to be introduced may increase its expression level by stabilizing the transcript and allowing its effective translocation out of the nucleus. Among the known such intron sequences are the introns of the plant ubiquitin gene (Cornejo, *Plant Mol. Biol. 23*, 567-581, 1993). Furthermore, it has been observed that the same construct inserted at different loci on the genome can vary in the level of expression in plants. The effect is believed to be due at least in part to the position of the gene on the chromosome, *i.e.*, individual isolates will have different expression levels (see, for example, Hoever et al.,

Transgenic Res. 3, 159-166, 1994). Further regulatory DNA
sequences that may be used for the construction of expression
cassettes include, for example, sequences that are capable of
regulating the transcription of an associated DNA sequence in
5 plant tissues in the sense of induction or repression.

There are, for example, certain plant genes that are known to
be induced by various internal and external factors, such as
plant hormones, heat shock, chemicals, pathogens, oxygen
10 deficiency, light, stress, etc.

A further group of DNA sequences which can be regulated
comprises chemically-driven sequences that are present, e.g.,
in the PR (pathogenesis-related) protein genes of tobacco and
15 are inducible by means of chemical regulators such as those
described in EP-A 0 332 104.

Yet another consideration in expression of foreign genes in
plants, animals, insects, fungi or microorganims is the level
20 of stability of the transgenic genome, *i.e.*, the tendency of
a foreign gene to segregate from the population. If a
selectable marker is linked to the gene or expression
cassette of interest, then selection can be applied to
maintain the transgenic host organism or part thereof.

25

It may be beneficial to include 5' leader sequences in the
expression cassette construct. Such leader sequences can act
to enhance translation. Translation leaders are known in the
art and include: picornavirus leaders, for example, EMCV
30 leader (Encephalomyocarditis 5' noncoding region; Elroy-Stein
et al., *Proc. Natl. Acad. Sci. USA 86*, 6126-6130, 1989);
potyvirus leaders, for example, TEV leader (Tobacco Etch
Virus; Allisson et al., *Virology 154,* 9-20, 1986); and human

immunoglobulin heavy-chain binding protein (BiP, Macejak and Sarnow, *Nature 353*, 90-94, 1991); untranslated leader from the coat protein mRNA of alfalfa mosaic virus (AMV RNA 4; Jobling and Gehrke, *Nature 325*, 622-625, 1987); tobacco

5 mosaic virus leader (TMV; Gallie et al., *Molecular Biology of RNA*, 237-256, 1989); and maize chlorotic mottle virus leader (MCMV; Lommel et al., *Virology 81*, 382-385, 1991; see also, Della-Cioppa et al., 1987, s.a.).

10 Depending upon where the DNA sequence encoding protamine is to be expressed, it may be desirable to synthesize the sequence with host preferred codons, or alternatively with chloroplast or plastid preferred codons. The plant preferred codons may be determined from the codons of highest frequency

15 in the proteins expressed in the largest amount in the particular plant species of interest (see, EP-A 0 359 472; EP-A 0 386 962; WO 91/16432; Perlak et al., *Proc. Natl. Acad. Sci 88*, 3324-3328, 1991; and Murray et al., *Nucl. Acids. Res. 17*, 477-498, 1989). In this manner, the nucleotide sequences

20 can be optimized for expression in any targeted host. It is recognized that all or any part of the gene sequence may be optimized or synthetic. That is, synthetic or partially optimized sequences may also be used. For the construction of chloroplast preferred genes, see USPN 5,545,817.

25 Expression systems encoding protamine are useful for the study of protamine activity, particularly in the context of transgenic cells, tissues or animals. Preferred is a system in which protamine expression has been attenuated,

30 particularly where this is achieved by means of transposon insertion. Mutant cells, tissues or animals according to the invention have impaired protamine expression. Especially those expression mutants in which expression is severely

attenuated but not limited, are useful for the study of protamine activity. They show increased sensitivity to modulated interaction of putative upstream signalling agents with specific target domains of protamine, as well as

5   modification of the downstream targets predicted to mediate its biological response. Thus, the invention also provides a method for assessing the ability of an agent to target protamine activity comprising exposing a protamine mutant as described herein to the agent, and judging the effect of the

10 biological activity of protamine.

In preparing the transcription cassette, the various DNA fragments may be manipulated, so as to provide for the DNA sequences in the proper orientation and, as appropriate in

15 the proper reading frame. Towards this end, adapters or linkers may be employed to join the DNA fragments or other manipulations may be involved to provide for convenient restriction sites, removal of superfluous DNA, removal of restriction sites, or the like. For this purpose, *in vitro*

20 mutagenesis, primer repair, restriction, annealing, resection, ligation, or the like may be employed, where insertions, deletions or substitutions, e.g. transitions and transversions, may be involved.

25 The expression cassette carrying the cDNA or genomic DNA encoding native or mutant protamine is placed into an expression vector by standard methods. As used herein, vector (or plasmid) refers to discrete elements that are used to introduce heterologous DNA into cells for either expression

30 or replication thereof. Selection and use of such vehicles are well within the skill of the artisan. Many vectors are available, and selection of an appropriate vector will depend on the intended use of the vector, i.e. whether it is to be

used for DNA amplification or for DNA expression, the size of the DNA to be inserted into the vector, the type of host (plant, animal, insect, fungi or microorganism) to be transformed with the vector, and the method of introducing

5   the expression vector into host cells. Each vector contains various components depending on its function (amplification of DNA or expression of DNA) and the host cell for which it is compatible. A typical expression vector generally includes, but is not limited to, prokaryotic DNA elements

10   coding for a bacterial replication origin and an antibiotic resistance gene to provide for the growth and selection of the expression vector in the bacterial host; a cloning site for insertion of an exogenous DNA sequence, which in this context would code for a protamine polypeptide or functional

15   fragment thereof; prokaryotic or eukaryotic DNA elements that control initiation of transcription of the exogenous gene, such as a promoter; and DNA elements that control the processing of transcripts, such as a transcription termination/polyadenylation sequence. It also can contain

20   such sequences as are needed for the eventual integration of the vector into the chromosome of the targeted host.

If prokaryotes are selected for recombinant production of protamine, it is preferred to use an inducible promoter, such

25   as for e.g. pelB (accession no. M17364) and ompT (accession no. X06903).

In a preferred embodiment, the expression vector also contains a gene encoding a selection marker such as, e.g.

30   hygromycin phosphotransferase (van den Elzen et al., *Plant Mol. Biol.* 5, 299-392, 1985), which is functionally linked to a promoter. Additional examples of genes that confer antibiotic resistance and are thus suitable as selectable

markers include those coding for neomycin phosphotransferase kanamycin resistance (Velten et al., *EMBO J. 3*, 2723-2730, 1984); the kanamycin resistance (NPT II) gene derived from Tn5 (Bevan et al., *Nature 304*, 184-187, 1983); the PAT gene

5   described in Thompson et al., (*EMBO J. 6*, 2519-2523, 1987); and chloramphenicol acetyltransferase. For a general description of plant expression vectors and selectable marker genes suitable according to the present invention, see Gruber et al., [in: *Methods in Plant Molecular Biology and*

10 *Biotechnology* 89-119 (CRC Press), 1993]. As to a selective gene marker appropriate for yeast, any marker gene can be used which facilitates the selection for transformants due to the phenotypic expression of the marker gene. Suitable markers for yeast are, for example, those conferring

15 resistance to antibiotics G418, hygromycin or bleomycin, or provide for prototrophy in an auxotrophic yeast mutant, for example the URA3, LEU2, LYS2, TRP1, or HIS3 gene.

Suitable selectable markers for mammalian cells are those

20 that enable the identification of cells competent to take up protamine nucleic acid, such as dihydrofolate reductase (DHFR, methotrexate resistance), thymidine kinase, or genes conferring resistance to G418 or hygromycin. The mammalian cell transformants are placed under selection pressure which

25 only those transformants which have taken up and are expressing the marker are uniquely adapted to survive. In the case of a DHFR or glutamine synthase (GS) marker, selection pressure can be imposed by culturing the transformants under conditions in which the pressure is progressively increased,

30 thereby leading to amplification (at its chromosomal integration site) of both the selection gene and the linked DNA that encodes protamine. Amplification is the process by which genes in greater demand for the production of a protein

critical for growth, together with closely associated genes which may encode a desired protein, are reiterated in tandem within the chromosomes of recombinant cells. Increased quantities of desired protein are usually synthesised from

5   thus amplified DNA.

A promoter element employed to control expression of the gene of interest and the marker gene, respectively, can be any plant-compatible promoter. Those can be plant gene promoters,

10 such as the promoter for the small subunit of ribulose-1,5-bis-phosphate carboxylase (RUBISCO), or promoters from tumour-inducing plasmids of *Agrobacterium tumefaciens*, like that nopaline synthase and octopine synthase promoters, or viral promoters such as the cauliflower mosaic virus (CaMV)

15 19S and 35S promoters or the figwort mosaic virus 35S promoter. See international application WO 91/19806, for example, for a review of known plant promoters which are suitable for use in the present invention.

20 According to a preferred embodiment, the cassette for the expression of protamine comprises the protamine encoding nucleic acid sequence translationally fused to a sequence encoding a transit peptide, e.g. for plastid import, polyadenylation signals and transcription terminators, each

25 operably linked to a suitable constitutive, inducible or tissue-specific promoter which enables the expression of the desired protein in plant cells, seeds, tissues or in whole plants.

30 Moreover, the protamine gene according to the invention preferably includes a signal peptide, leader or secretion sequence in order to facilitate secretion of the polypeptide from bacterial hosts, such that it will be produced as a

soluble native peptide rather than in an inclusion body. Examples for such leader sequences include pelB (accession no. M17364) and ompT (accession no. X06903). The peptide can be recovered from the bacterial periplasmic space, or the
5 culture medium, as appropriate.

Suitable promoting sequences for use with yeast hosts may be regulated or constitutive and are preferably derived from a highly expressed yeast gene, especially a Saccharomyces
10 cerevisiae gene. Thus, the promoter of the TRP1 gene, the ADHI or ADHII gene, the acid phosphatase (PHO5) gene, a promoter of the yeast mating pheromone genes coding for the alpha- or a-factor or a promoter derived from a gene encoding a glycolytic enzyme such as the promoter of the enolase,
15 glyceraldehyde-3-phosphate dehydrogenase (GAP), 3-phospho-glycerate kinase (PGK), hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phos-phoglycerate mutase, pyruvate kinase, triose phosphate isomerase, phosphoglucose isomerase or glucokinase genes, or
20 a promoter from the TATA binding protein (TBP) gene can be used. Furthermore, it is possible to use hybrid promoters comprising upstream activation sequences (UAS) of one yeast gene and downstream promoter elements including a functional TATA box of another yeast gene, for example a hybrid promoter
25 including the UAS(s) of the yeast PHO5 gene and downstream promoter elements including a functional TATA box of the yeast GAP gene (PHO5-GAP hybrid promoter). A suitable constitutive PHO5 promoter is e.g. a shortened acid phosphatase PHO5 promoter devoid of the upstream regulatory
30 elements (UAS) such as the PHO5 (-173) promoter element starting at nucleotide -173 and ending at nucleotide -9 of the PHO5 gene.

Protamine gene transcription from vectors in mammalian hosts
may be controlled by promoters derived from the genomes of
viruses such as polyoma virus, adenovirus, fowlpox virus,
bovine papilloma virus, avian sarcoma virus, cytomegalovirus
5   (CMV), a retrovirus and Simian Virus 40 (SV40), from
heterologous mammalian promoters such as the actin promoter
or a very strong promoter, e.g. a ribosomal protein promoter,
and from the promoter normally associated with protamine
sequence, provided such promoters are compatible with the
10   host cell systems.

Transcription of a DNA encoding protamine by higher
eukaryotes may be increased by inserting an enhancer sequence
into the vector. Enhancers are relatively orientation and
15   position independent. Many enhancer sequences are known from
mammalian genes (e.g. elastase and globin). However,
typically one will employ an enhancer from a eukaryotic cell
virus. Examples include the SV40 enhancer on the late side of
the replication origin (bp 100-270) and the CMV early
20   promoter enhancer. The enhancer may be spliced into the
vector at a position 5' or 3' to protamine DNA, but is
preferably located at a site 5' from the promoter.

Advantageously, a eukaryotic expression vector encoding
25   protamine can comprise a locus_control_region (LCR). LCRs are
capable of directing high-level integration site independent
expression of transgenes integrated into host cell chromatin,
which is of importance especially where the protamine gene is
to be expressed in the context of a permanently-transfected
30   eukaryotic cell line in which chromosomal integration of the
vector has occurred, in vectors designed for gene therapy

applications or in transgenic animals or other hosts disclosed herein or known in the art.

According to a preferred embodiment of the present invention,
5 the expression cassettes and plasmid or vector systems disclosed herein additionally comprise nucleic acid sequences which encode specific protamine modifying enzymes and/or protamine binding proteins, preferably being co-expressed with the polypeptide according to the invention, as already
10 outlined above.

Suitable eukaryotic host cells for expression of protamine embrace fungi including yeast, insect, plant, animal, human, or nucleated cells from other multicellular organisms will
15 also contain sequences necessary for the termination of transcription and for stabilising the mRNA. Such sequences are commonly available from the 5' and 3' untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated
20 fragments in the untranslated portion of the mRNA encoding protamine.

The procaryotic or eucaryotic host cells, seeds, tissues and whole organisms contemplated in the context of the present
25 - invention may be obtained by any of several methods. Those skilled in the art will appreciate that the choice of method might depend on the type of host such as plant, i.e. monocot or dicot, targeted for transformation. Such methods generally include direct gene transfer, chemically-induced gene
30 transfer, electroporation, microinjection (Crossway et al., *BioTechniques* 4, 320-334, 1986; Neuhaus et al., *Theor. Appl. Genet.* 75, 30-36, 1987), Agrobacterium-mediated gene

transfer, ballistic particle acceleration using, for example, devices available from Agracetus, Inc., Madison, Wisconsin, and Dupont, Inc., Wilmington, Delaware (see, for example, Sanford et al., U.S. Patent 4,945,050; and Mc Cabe et al., 5 *Biotechnology 6*, 923-926, 1988), and the like.

When introducing a chosen gene construct into a cell, certain considerations must be taken into account, well known to those skilled in the art. The nucleic acid to be inserted 10 should be assembled within a construct which contains effective regulatory elements which will drive transcription. There must be available a method of transporting the construct into the cell. Once the construct is within the cell membrane, integration into the endogenous chromosomal 15 material either will or will not occur. Finally, as far as plants are concerned the target cell type may be such that cells can be regenerated into whole plants.

The particular choice of a transformation technology will be 20 determined by its efficiency to transform certain plant species as well as the experience and preference of the person practising the invention with a particular methodology of choice. It will be apparent to the skilled person that the particular choice of a transformation system to introduce 25 nucleic acid into plant cells is not essential to or a limitation of the invention, nor is the choice of technique for plant regeneration.

One method for obtaining the present transformed plants or 30 parts thereof is direct gene transfer in which plant cells are cultured or otherwise grown under suitable conditions in the presence of DNA oligonucleotides comprising the nucleotide sequence desired to be introduced into the plant

or part thereof. The donor DNA source is typically a plasmid or other suitable vector containing the desired gene or genes. For convenience, reference is made herein to plasmids, with the understanding that other suitable vectors containing

5   the desired gene are also contemplated.

Any suitable plant tissue which takes up the plasmid may be treated by direct gene transfer. Such plant tissue includes, for example, reproductive structures at an early stage of

10   development, particularly prior to meiosis, and especially 1-2 weeks pre-meiosis. Generally, the pre-meiotic reproductive organs are bathed in plasmid solution, such as, for example, by injecting plasmid solution directly into the plant at or near the reproductive organs. The plants are then self-

15   pollinated, or cross-pollinated with pollen from another plant treated in the same manner. The plasmid solution typically contains about 10-50 µg DNA in about 0.1-10 ml per floral structure, but more or less than this may be used depending on the size of the particular floral structure. The

20   solvent is typically sterile water, saline, or buffered saline, or a conventional plant medium. If desired, the plasmid solution may also contain agents to chemically induce or enhance plasmid uptake, such as, for example, PEG, Ca^{2+} or the like.

25   Following exposure of the reproductive organs to the plasmid, the floral structure is grown to maturity and the seeds are harvested. Depending on the plasmid marker, selection of the transformed plants with the marker gene is made by

30   germination or growth of the plants in a marker-sensitive, or preferably a marker-resistant medium. For example, seeds obtained from plants treated with plasmids having the kanamycin resistance gene will remain green, whereas those

without this marker gene are albino. Presence of the desired gene transcription of mRNA therefrom and expression of the peptide can further be demonstrated by conventional Southern, northern, and western blotting techniques.

All plant transformation systems produce a mixture of transgenic and non-transgenic plants. The selection of transgenic plant cells can be accomplished by the introduction of an antibiotic or herbicide gene, enabling the transgenic plant cells to be selected on media containing the corresponding toxic compound. Besides those marker systems for the selection of transgenic plants new so-called "positive selection systems" have been successfully used for plant transformation (PCT/EP94/00575, WO94/20627). In contrast to antibiotic or herbicide resistance selection systems in which transgenic cells acquire the ability to survive on a selection medium while non-transgenic cells are killed, this method favours regeneration and growth of the transgenic plant cells while non-transgenic plant cells are starved, but not killed. Therefore, this selection strategy is termed "positive selection". Vector systems for Agrobacterium-mediated transformation have been constructed and have been successfully used e.g. to transform potato, tobacco and tomato and are described e.g. by Haldrup, A., Petersen S.G. and Okkels F.T. [Plant Mol. Biol. 37, pp. 287-296, (1998)]. Transformtion systems based on this positive selection systems can be used according to the invention to introduce constructs harbouring protamine to obtain plants expressing the protamine polypeptide or functional fragment thereof. In addition, the use of those selection systems would have the advantage to overcome disadvantages in using antibiotic or herbicide genes in a selection system such as e.g. toxicity or allergenicity of the gene product and

interference with antibiotic treatment, as generally known in the art.

The list of possible transformation methods given above by
5   way of example is not claimed to be complete and is not intended to limit the subject of the invention in any way.

The present invention therefore also comprises a procaryotic or eucaryotic host cell, seed, tissue or whole organism
10 transformed or transfected with the DNA molecule or with the plasmid or vector system according to the invention as set out hereinbefore in a manner enabling said host cell, seed, tissue or whole organism to express a polypeptide or functional fragment thereof having the biological activity of
15 protamine and/or having the capability of specifically binding to antibodies raised against said polypeptide or functional fragment thereof.

According to the invention, the procaryotic or eucaryotic
20 host cell, seed, tissue or whole organism is selected from the group consisting of bacteria, yeast, fungi, insect, animal and plant cells, seeds, tissues or whole organisms. As for the procaryotic taxonomic groups, the host can be selected from the group consisting of proteobacteria
25 including members of the alpha, beta, gamma, delta and epsilon subdivision, gram-positive bacteria including Actinomycetes, Firmicutes, Clostridium and relatives, flavobacteria, cyanobacteria, green sulfur bacteria, green non-sulfur bacteria, and archaea. Suitable proteobacteria
30 belonging to the alpha subdivision can be selected from the group consisting of Agrobacterium, Rhodospirillum, Rhodopseudomonas, Rhodobacter, Rhodomicrobium, Rhodopila, Rhizobium, Nitrobacter, Aquaspirillum, Hyphomicrobium,

Acetobacter, Beijerinckia, Paracoccus and Pseudomonas, with Rhodopseudomonas and Pseudomonas being preferred and Rhodopseudomonas palustris and Pseudomonas fluorescens, respectively, being most preferred. Suitable proteobacteria belonging to the beta subdivision can be selected from the group consisting of Rhodocyclus, Rhodopherax, Rhodovivax, Spirillum, Nitrosomonas, Spherotilus, Thiobacillus, Alcaligenes, Pseudomonas, Bordetella and Neisseria, with ammonia-oxidizing bacteria such as Nitrosomonas being preferred and Nitrosomonas sp. ENI-11 being most preferred. Suitable proteobacteria belonging to the gamma subdivision can be selected from the group consisting of Chromatium, Thiospirillum, Beggiatoa, Leucothrix, Escherichia and Azotobacter, with Enterobacteriaceae such as Escherichia coli being preferred, and with E. coli K12 strains such as e.g. M15 (described as DZ 291 by Villarejo et al. in J. Bacteriol. 120, 466-474, 1974), HB 101 (ATCC No. 33649) and E. coli SG13009 (Gottesman et al., J. Bacteriol. 148, 265-273, 1981) being most preferred. Suitable proteobacteria belonging to the delta subdivision can be selected from the group consisting of Bdellovibrio, Desulfovibrio, Desulfuromonas and Myxobacteria such as Myxococcus, with Myxococcus xanthus being preferred. Suitable proteobacteria belonging to the epsilon subdivision can be selected from the group consisting of Thiorulum, Wolinella and Campylobacter. Suitable gram-positive bacteria can be selected from the group consisting of Actinomycetes such as Actinomyces, Bifidobacterium, Propionibacterium, Strepto-myces, Nocardia, Actinoplanes, Arthrobacter, Coryne-bacterium, Mycobacterium, Micromonospora, Frankia, Cellulomonas and Brevibacterium, and Firmicutes including Clostridium and relatives such as Clostridium, Bacillus, Desulfotomaculum, Thermoactinomyces,

Sporosarcina, Acetobac-terium, Streptococcus, Enterococcus, Peptococcus, Lacto-bacillus, Lactococcus, Staphylococcus, Rominococcus, Plano-coccus, Mycoplasma, Acheoleplasma and Spiroplasma, with Bacillus subtilis and Lactococcus lactis

5   being preferred. Suitable flavobacteria can be selected from the group consisting of Bacteroides, Cytophaga and Flavobacterium, with Flavobacterium such as Flavobacterium ATCC21588 being preferred. Suitable cyanobacteria can be selected from the group consisting of Chlorococcales

10 including Synecho-cystis and Synechococcus, with Synechocystis sp. and Synechococcus sp. PS717 being preferred. Suitable green sulfur bacteria can be selected from the group Chlorobium, with Chlorobium limicola f. thiosulfatophilum being preferred. Suitable green non-sulfur

15 bacteria can be selected from the group Chloroflexaceae such as Chloroflexus, with Chloroflexus aurantiacus being preferred. Suitable archaea can be selected from the group of Halobacteriaceae including Halobacterium, with Halobacterium salinarum being preferred.

20
As for the eucaryotic taxonomic group of fungi including yeast, the host can be selected from the group consisting of Ascomycota including Saccharomycetes such as Pichia and Saccharomyces, and anamorphic Ascomycota including

25 Aspergillus, with Saccharomyces cerevisiae and Aspergillus niger (e.g. ATCC 9142) being preferred.

The eucaryotic host system comprises insect cells which preferably are selected from the group consisting of SF9,

30 SF21, Trychplusiani and MB21. For example, the polypeptides according to the invention can advantageously be expressed in insect cell systems. Insect cells suitable for use in the method of the invention include, in principle, any

lepidopteran cell which is capable of being transformed with an expression vector and expressing heterologous proteins encoded thereby. In particular, use of the Sf cell lines, such as the *Spodoptera frugiperda* cell line IPBL-SF-21 AE

5   (Vaughn *et al.*, (1977) In Vitro <u>13</u>, 213-217) is preferred. The derivative cell line Sf9 is particularly preferred. However, other cell lines, such as *Tricoplusia ni* 368 (Kurstack and Marmorosch, (1976) Invertebrate Tissue Culture Applications in Medicine, Biology and Agriculture. Academic

10 Press, New York, USA) can be employed. These cell lines, as well as other insect cell lines suitable for use in the invention, are commercially available (e.g. from Stratagene, La Jolla, CA, USA). As well as expression in insect cells in culture, the invention also comprises the expression of

15 heterologous proteins such as protamine in whole insect organisms. The use of virus vectors such as baculovirus allows infection of entire insects, which are in some ways easier to grow than cultured cells as they have fewer requirements for special growth conditions. Large insects,

20 such as silk moths, provide a high yield of heterologous protein. The protein can be extracted from the insects according to conventional extraction techniques. Expression vectors suitable for use in the invention include all vectors which are capable of expressing foreign proteins in insect

25 cell lines. In general, vectors which are useful in mammalian and other eukaryotic cells are also applicable to insect cell culture. Baculovirus vectors, specifically intended for insect cell culture, are especially preferred and are widely obtainable commercially (e.g. from Invitrogen and Clontech).

30 Other virus vectors capable of infecting insect cells are known, such as Sindbis virus (Hahn *et al.*, (1992) PNAS (USA) <u>89</u>, 2679-2683). The baculovirus vector of choice (reviewed by

Miller (1988) Ann. Rev. Microbiol. <u>42</u>, 177-199) is *Autographa californica* multiple nuclear polyhedrosis virus, AcMNPV. Typically, the heterologous gene replaces at least in part the polyhedrin gene of AcMNPV, since polyhedrin is not required for virus production. In order to insert the heterologous gene, a transfer vector is advantageously used. Transfer vectors are prepared in *E. coli* hosts and the DNA insert is then transferred to AcMNPV by a process of homologous recombination.

The eucaryotic host sytem further comprises animal cells preferably selected from the group consisting of Baby Hamster Kidney (BHK) cells, Chinese Hamster Ovarian (CHO) cells, Human Embryonic Kidney (HEK) cells and COS cells, with NIH 3T3 and 293 being most preferred..

The host cells referred to in this disclosure comprise cells in *in vitro* culture as well as cells that are within a host organism.

The present invention also provides transgenic plant material, selected from the group consisting of protoplasts, cells, calli, tissues, organs, seeds, embryos, ovules, zygotes, etc. and especially, whole plants, that has been transformed by means of the method according to the invention and comprises the recombinant DNA of the invention in expressible form, and processes for the production of the said transgenic plant material.

In accordance with yet another embodiment of the present invention, there are provided antibodies specifically recognising and binding to protamine. For example, such antibodies may be generated against a protamine polypeptide

species having any of the amino acid sequences as set forth
in SEQ ID NOS. 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24,
and 34. Alternatively, protamine or protamine fragments
(which may also be synthesised by *in vitro* methods), such as
5   those described hereinbefore, are fused (by recombinant
expression or an *in vitro* peptidyl bond) to an immunogenic
polypeptide, and this fusion polypeptide, in turn, is used to
raise antibodies against a protamine epitope.

10 Anti-protamine antibodies may be recovered from the serum of
immunised animals. Monoclonal antibodies may be prepared from
cells from immunised animals in the conventional manner.

The antibodies of the invention are useful for studying
15 protamine localisation, screening of an expression library to
identify nucleic acids encoding protamine or the structure of
functional domains, as well as for the purification of
protamine, and the like.

20 Antibodies according to the invention may be whole antibodies
of natural classes, such as IgE and IgM antibodies, but are
preferably IgG antibodies. Moreover, the invention includes
antibody fragments, such as Fab, F(ab')$_2$, Fv and ScFv. Small
fragments, such Fv and ScFv, possess advantageous properties
25 for diagnostic and therapeutic applications on account of
their small size and consequent superior tissue distribution.

The antibodies according to the invention are especially
indicated for diagnostic and therapeutic applications.
30 Accordingly, they may be altered antibodies comprising an
effector protein such as a toxin or a label. Especially
preferred are labels which allow the imaging of the

distribution of the antibody in a tumour *in vivo*. Such labels may be radioactive labels or radioopaque labels, such as metal particles, which are readily visualisable within the body of a patient. Moreover, the may be fluorescent labels or

5   other labels which are visualisable on tissue samples removed from patients. In addition, said antibodies can also be used for purifying protamine from native sources or from transformed host cells or culture medium.

10 Recombinant DNA technology may be used to improve the antibodies of the invention. Thus, chimeric antibodies may be constructed in order to decrease the immunogenicity thereof in diagnostic or therapeutic applications. Moreover, immunogenicity may be minimised by humanising the antibodies

15 by CDR grafting [see EP-A 0 239 400 (Winter)] and, optionally, framework modification [see WO 90/07861 (Protein Design Labs)].

    Antibodies according to the invention may be obtained from

20 animal serum, or, in the case of monoclonal antibodies or fragments thereof, produced in cell culture. Recombinant DNA technology may be used to produce the antibodies according to established procedure, in bacterial or preferably mammalian cell culture. The selected cell culture system preferably

25 secretes the antibody product.

    Therefore, the present invention includes a process for the production of an antibody according to the invention comprising culturing a host, e.g. E. coli or a mammalian

30 cell, which has been transformed with a hybrid vector comprising an expression cassette comprising a promoter operably linked to a first DNA sequence encoding a signal

peptide linked in the proper reading frame to a second DNA sequence encoding the antibody, and isolating said antibody.

Multiplication of hybridoma cells or mammalian host cells *in*
5   *vitro* is carried out in suitable culture media, which are the customary standard culture media, for example Dulbecco's Modified Eagle Medium (DMEM) or RPMI 1640 medium, optionally replenished by a mammalian serum, e.g. fetal calf serum, or trace elements and growth sustaining supplements, e.g. feeder
10 cells such as normal mouse peritoneal exudate cells, spleen cells, bone marrow macrophages, 2-aminoethanol, insulin, transferrin, low density lipoprotein, oleic acid, or the like. Multiplication of host cells which are bacterial cells or yeast cells is likewise carried out in suitable culture
15 media known in the art, for example for bacteria in medium LB, NZCYM, NZYM, NZM, Terrific Broth, SOB, SOC, 2 x YT, or M9 Minimal Medium, and for yeast in medium YPD, YEPD, Minimal Medium, or Complete Minimal Dropout Medium.

20 *In vitro* production provides relatively pure antibody preparations and allows scale-up to give large amounts of the desired antibodies. Techniques for bacterial cell, yeast or mammalian cell cultivation are known in the art and include homogeneous suspension culture, e.g. in an airlift reactor or
25 in a continuous stirrer reactor, or immobilised or entrapped cell culture, e.g. in hollow fibres, microcapsules, on agarose microbeads or ceramic cartridges.

Large quantities of the desired antibodies can also be
30 obtained by multiplying mammalian cells *in vivo*. For this purpose, hybridoma cells producing the desired antibodies are injected into histocompatible mammals to cause growth of

antibody-producing tumours. Optionally, the animals are primed with a hydrocarbon, especially mineral oils such as pristane (tetramethyl-pentadecane), prior to the injection. After one to three weeks, the antibodies are isolated from the body fluids of those mammals. For example, hybridoma cells obtained by fusion of suitable myeloma cells with antibody-producing spleen cells from Balb/c mice, or transfected cells derived from hybridoma cell line Sp2/0 that produce the desired antibodies are injected intraperitoneally into Balb/c mice optionally pre-treated with pristane, and, after one to two weeks, ascitic fluid is taken from the animals.

The cell culture supernatants are screened for the desired antibodies, preferentially by immunofluorescent staining of cells expressing protamine, by immunoblotting, by an enzyme immunoassay, e.g. a sandwich assay or a dot-assay, or a radioimmunoassay.

For isolation of the antibodies, the immunoglobulins in the culture supernatants or in the ascitic fluid may be concentrated, e.g. by precipitation with ammonium sulphate, dialysis against hygroscopic material such as polyethylene glycol, filtration through selective membranes, or the like. If necessary and/or desired, the antibodies are purified by the customary chromatography methods, for example gel filtration, ion-exchange chromatography, chromatography over DEAE-cellulose and/or (immuno-)affinity chromatography, e.g. affinity chromatography with protamine protein or with Protein-A.

The invention further concerns hybridoma cells secreting the monoclonal antibodies of the invention. The preferred hybridoma cells of the invention are genetically stable, secrete monoclonal antibodies of the invention of the desired
5   specificity and can be activated from deep-frozen cultures by thawing and recloning.

The invention also concerns a process for the preparation of a hybridoma cell line secreting monoclonal antibodies
10   directed against protamine, characterised in that a suitable mammal, for example a Balb/c mouse, is immunised with purified protamine protein, an antigenic carrier containing purified protamine or with cells bearing protamine, antibody-producing cells of the immunised mammal are fused with cells
15   of a suitable myeloma cell line, the hybrid cells obtained in the fusion are cloned, and cell clones secreting the desired antibodies are selected. For example spleen cells of Balb/c mice immunised with cells bearing protamine are fused with cells of the myeloma cell line PAI or the myeloma cell line
20   Sp2/0-Ag14, the obtained hybrid cells are screened for secretion of the desired antibodies, and positive hybridoma cells are cloned.

Preferred is a process for the preparation of a hybridoma
25   cell line, characterised in that Balb/c mice are immunised by injecting subcutaneously and/or intraperitoneally between 10 and 10^7 and 10^8 cells of human tumour origin which express protamine containing a suitable adjuvant several times, e.g. four to six times, over several months, e.g. between two and
30   four months, and spleen cells from the immunised mice are taken two to four days after the last injection and fused with cells of the myeloma cell line PAI in the presence of a

fusion promoter, preferably polyethylene glycol. Preferably the myeloma cells are fused with a three- to twentyfold excess of spleen cells from the immunised mice in a solution containing about 30 % to about 50 % polyethylene glycol of a

5   molecular weight around 4000. After the fusion the cells are expanded in suitable culture media as described hereinbefore, supplemented with a selection medium, for example HAT medium, at regular intervals in order to prevent normal myeloma cells from overgrowing the desired hybridoma cells.

10

The invention also concerns recombinant DNAs comprising an insert coding for a heavy chain variable domain and/or for a light chain variable domain of antibodies directed to the protamine protein. By definition such DNAs comprise coding

15   single stranded DNAs, double stranded DNAs consisting of said coding DNAs and of complementary DNAs thereto, or these complementary (single stranded) DNAs themselves.

Furthermore, DNA encoding a heavy chain variable domain

20   and/or for a light chain variable domain of antibodies directed against protamine can be enzymatically or chemically synthesised DNA having the authentic DNA sequence coding for a heavy chain variable domain and/or for the light chain variable domain, or a mutant thereof. A mutant of the

25   authentic DNA is a DNA encoding a heavy chain variable domain and/or a light chain variable domain of the above-mentioned antibodies in which one or more amino acids are deleted or exchanged with one or more other amino acids. Preferably said modification(s) are outside the CDRs of the heavy chain

30   variable domain and/or of the light chain variable domain of the antibody. Such a mutant DNA is also intended to be a silent mutant wherein one or more nucleotides are replaced by

other nucleotides with the new codons coding for the same amino acid(s). Such a mutant sequence is also a degenerated sequence. Degenerated sequences are degenerated within the meaning of the genetic code in that an unlimited number of

5 nucleotides are replaced by other nucleotides without resulting in a change of the amino acid sequence originally encoded. Such degenerated sequences may be useful due to their different restriction sites and/or frequency of particular codons which are preferred by the specific host,

10 particularly E. coli, to obtain an optimal expression of the heavy chain murine variable domain and/or a light chain murine variable domain.

The term "mutant" is intended to include a DNA mutant

15 obtained by in vitro mutagenesis of the authentic DNA according to methods known in the art.

For the assembly of complete tetrameric immunoglobulin molecules and the expression of chimeric antibodies, the

20 recombinant DNA inserts coding for heavy and light chain variable domains are fused with the corresponding DNAs coding for heavy and light chain constant domains, then transferred into appropriate host cells, for example after incorporation into hybrid vectors.

25

In the case of a diagnostic composition, the antibody is preferably provided together with means for detecting the antibody, which may be enzymatic, fluorescent, radioisotopic or other means. The antibody and the detection means may be

30 provided for simultaneous, simultaneous separate or sequential use, in a diagnostic kit intended for diagnosis.

In a further aspect of the invention there is provided a
pharmaceutical composition comprising a bioactive protamine
protein, peptide or a functionally equivalent molecule or a
combination thereof displaying protamine activity, and
5   optionally a pharmaceutically acceptable carrier or
excipient. Compositions comprising such carriers can be
formulated by well known conventional methods.

For parenteral administration, the protamine proteins,
10 polypeptides or functional fragments thereof (as defined
herein) can be, for example, formulated as a solution,
suspension, or lyophilised powder in association with a
pharmaceutically acceptable parenteral vehicle. Examples of
such vehicles are water, saline, Ringer's solution, dextrose
15. solution, and 5% human serum albumin. The vehicle or
lyophilised powder may contain additives that maintain
isotonicity (e.g. sodium chloride, mannitol) and chemical
stability (e.g. buffers and preservatives). For example, a
parenteral composition suitable for administration by
20 injection may be prepared by dissolving 1.5% by weight of
active ingredient in 0.9% sodium chloride solution. The
formulation may be sterilised by any commonly used technique.

The pharmaceutical composition according to the invention may
25 be administered as a single dose or in multiple doses. The
said compositions may be administered either as individual
therapeutic agents or in combination with each other or with
other therapeutic agents. The treatments of the present
invention may be combined with conventional therapies which
30 may be administered sequentially or simultaneously.

The pharmaceutical compositions of the invention may be administered by any means that enables the active protamine protein component to reach the targeted cells. Since compounds of the invention may be susceptible to digestion enzymes found in the lumen and/or linings of the gut, when administered orally, parenteral administration i.e. via intravenous, subcutaneous, or intramuscular would appear to be the preferred route of administration. Intravenous administration may be accomplished with the aid of an infusion pump. Alternatively, the protamine protein component can be formulated as aerosol medicaments for intranasal inhalation.

The dosage required varies depending on factors such as pharmacodynamic characteristics; its mode and route of administration; age; health; and weight of the recipient; nature and extent of symptoms; type of concurrent treatment; and frequency of treatment. The dosage of the protamine component may be from 1 to 3000mgs/50kg of body weight; from 10 to 1000mgs/50kg of body weight; 25 to 800mgs/50kg of body weight, depending on the circumstances of the recipient in need of treatment thereof.

In a further aspect of the invention, the pharmaceutical composition comprising a protamine protein, peptide or a functionally equivalent molecule or a combination thereof additionally may comprise an anti-viral drug. Such a combination may further enhance the effectiveness of compositions of the invention and/or reduce undesired side effects. Examples of suitable anti-viral drugs are described, for example, by Flechter, Am. J. Hosp. Pharm. 51 (1994), 2251

- 2267 and by Stein et al., Clin. Inf. Dis. 17 (1993), 749 - 771.

A further embodiment of the present invention is the use of a
protamine protein, peptide or a functionally equivalent
molecule or a combination thereof for the preparation of a
pharmaceutical composition for the prophylaxis or the
treatment of diseases or malfunctions associated with heparin
or insulin binding. Included in this aspect of the invention
is the use of said protamine protein, peptide or a
functionally equivalent molecule or a combination thereof in
combination with an additional active ingredient.

According to a further aspect, the present invention relates
to a composition capable of killing microbial cells or
inhibiting growing microbial cells, i.e. bacteriocidal,
bacteriostatic, fungicidal and/or fungistatic composition,
which can be used for killing or inhibiting microbial cells
present on a hard surface, on skin or in laundry, and for
preserving food products, cosmetics, etc. Accordingly, the
present invention provides a cleaning, detergent or
disinfecting composition.

As discussed before, disinfectant formulations often contain
harmful additives like Tris-hydroxymethyl-aminomethane (Tris)
or Ethylene-diamine-tetra-acetic acid (EDTA). These
substances are known to enhance the permeability of bacterial
membranes or to form complexes with divalent cations which
are necessary for the stabilization of the bacterial cell
wall (VAARA 1992).

For purposes such as disinfection of small or large surfaces,
e.g. food, laboratory benches, clinicical apparatus, floors

or rooms including areas for keeping livestock, traces of these harmful compounds remain on the surfaces. Contact with them e.g. by touching, inhalating and/or swallowing can cause short- or long term indisposition and/ or illness in animals
5   and humans beings.

Ecological as well as economical reasons demand the development of harmless substances and disinfectant formulations. A first step towards a sustainable product
10 development is the awareness that biological and harmless defense systems particularly against bacteria exist, e.g. in the human eye. Hence, these systems have been adapted to their purpose.

15 In the course of experiments which have lead to the present invention, a harmless biological buffer system was developed on the basis of human tear electrolyte components containing the minimum number of compounds while causing a maximum effect, i.e. enhancing bacterial permeability.
20
Furthermore, we studied the bactericidal effect of protamine in combination with the developed biological buffer system on gram-positive and gram-negative bacteria. The bactericidal effect was studied on bacteria grown in rich medium in
25 contrast to previous studies mainly using minimal medium.

The results of our studies will be discussed with respect to other publications on the inhibitory effect of protamine in minimal medium. Additional factors, e.g. the impact of pH-
30 value alterations and the impact of natural permeabilizing substances will also be referred to.

According to the invention, harmless compounds displaying the desired activity, i.e. enhancing bacterial permeability, were identified by testing modified human tear electrolytes. The respective experiments conducted have lead to the definition

5 of compositions having the afore-mentioned effect or activity. Due to their potential of killing microbial cells or inhibiting growing microbial cells the compositions provided by the present invention are suitable as cleaning or detergent compositions and can be used whenever it is desired

10 to exert a bacteriocidal, bacteriostatic, fungicidal and/or fungistatic effect. It is clear for a person skilled in the art that there is a very broad range of application fields including, but not limited to, preparation and storage of food and food constituents, beverages, cosmetics and

15 pharmaceuticals, medicinal and veterinary purposes, disinfection of surfaces and articles. The respective compounds as well as their absolute and relative amounts and concentrations can easily be selected in view of the specific application.

20
Accordingly, the present invention provides a bacteriocidal, bacteriostatic, fungicidal and/or fungistatic composition comprising or consisting essentially of citrate and bicarbonate. The component citrate includes citrate acid and

25 salts of citrate in their hydrated forms such as trisodium salt of citric acid dihydrate. Similarly, the component bicarbonate includes carbonic acid and salts of mono- or bicarbonate such as sodium bicarbonate.

30 AYRES et al. (1999) demonstrated that citric acid is an effective permeabilizer of bacterial membranes and enhances the efficiency of several antibiotics. Citric acid is a natural substance, cheap and easy to store, and degradable.

As a further component phosphate may be comprised inlcuding phoshoric acid and salts of phosphate like sodium phosphate.

5   According to another preferred embodiment, the composition additionally comprises a basic protein or peptide selected from the group consisting of protamines, protamine sulphates, defensins, magainins, mellitin, cecropins and protegrins, with protamines and protamine sulphates being particularly
10   preferred.

A suitable biologically active protamine or functional fragment thereof can be selected from the group consisting of proteins, polypeptides or peptides representing or comprising
15   amino acid sequences extending from position 9 to position 18, from position 9 to position 20, from position 9 to position 21, from position 9 to position 22, from position 9 to position 23, from position 10 to position 20, from position 10 to position 21, from position 10 to position 22,
20   from position 10 to position 23, and from position 10 to 24 of SEQ ID NO. 2; amino acid sequences as set out in SEQ ID NOS. 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, and 26; amino acid sequences derived from SEQ ID NO. 33; amino acid sequences being encoded by nucleic acid sequences as set out
25   in SEQ ID NOS. 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, and 25; and amino acid sequences being encoded by nucleic acid sequences as set out in SEQ ID NOS. 27, 28, 29, 30, 31, and 32.

According to a further aspect, the composition further comprises lysozyme or any other substance known to degrade the cell wall of bacteria.

5   In the afore-mentioned compositions, it is preferred that the components citrate and bicarbonate are present in a molar ratio of 4:1, and that their preferred amounts are at least 0.04 M and 0.01 M, respectively. It should be understood that the amount of each component may generally vary from 0.01 to
10   0.4 Mol or even higher, depending on the envisaged field of application. Accordingly, the compositions may comprise more than 0.04 Mol citrate, preferably 0.2 Mol citrate, more preferably 0.4 Mol citrate, and more than 0.01 Mol bicarbonate, preferably 0.05 Mol, more preferably 0.1 Mol
15   bicarbonate. The skilled artisan is able to calculate the weight in gram in order to obtain the desired composition.

In the case of a composition which additionally comprises phosphate, it is preferred that the components citrate,
20   bicarbonate and phosphate are present in a molar ratio of 2:1:1, and that their preferred amounts are at least 0.2 M, 0.1 M and 0.1 M, respectively.

If the composition comprises a basic protein or peptide, it
25   is preferred that this component is present in an amount of at least 0.1 µg. In case of using lysozyme or any other cell-wall degrading substance, it is preferred that this component is present in an amount of at least 0.1 µg.

30   For a broad range of applications, it may be desired to use the compositions according to the invention in liquid form (solution), e.g. by dissolving the components in water or

other useful solvents and/or admixing prefabricated or stock solutions each comprising one or more of the compounds in a suitable concentration. Accordingly, the present invention provides such compositions in liquid form, which, in the case

5   of citrate and bicarbonate being the components of choice, may be obtained by admixing an at least 0.01 mol/l citrate solution adjusted to pH 6.0 to 7.0 with an at least 0.01 mol/l bicarbonate solution in a ratio of 4:1 (vol./vol.).

10   An effect was observed when a solution comprising citrate at a pH of 7.0 was mixed with a solution comprising bicarbonate. The ph shifted to about 9.5 and the solution caused a baceriocidal/fungicidal effect. This effect could also be shown when mixing a solution of citrate with a pH of 6.2 with

15   a solution comprising bicarbonate causing rise of the pH to about 8.0. Thus, the preferred pH for the citrate solution before mixing ranges between 6.0 and 7.0. In a preferred embodiment of the invention the solution after mixing comprises 0.4 mol/l citrate and 0.1 mol/l bicarbonate at a pH

20   of 8.0 to 9.5.

In the case of phosphate being additionally selected as active component, said solution may be obtained by admixing an at least 0.01 mol/l citrate solution adjusted to pH 6.0 to

25   7.0 with an at least 0.01 mol/l bicarbonate solution and an at least 0.01 mol/l phosphate solution adjusted to ph 6.0 to 7.0 in a ratio of 2:1:1 (vol./vol.).

The effects mentioned above could also be observed when

30   mixing a solution of citrate with a pH of 7.0 with a solution comprising bicarbonate and a solution comprising phosphate

with a pH of 7.0. The pH shifted to about 10.0 and the solution caused a bacteriocidal/fungicidal effect.

Similar effects were observed when mixing a solution of at
5   least 0.02 mol/l citrate with a pH of 6.2 with a solution comprising at least 0.01 mol/l bicarbonate and a solution comprising at least 0.01 mol/l phosphate with a pH of 6.2. The pH of the final solution shifted to about 8.0 and the solution caused a bacteriostatic/fungistatic effect.
10

In a preferred embodiment of the invention the final solution comprises 0.2 mol/l citrate and 0.1 mol/l bicarbonate and 0.1 mol/l phosphate at a pH ranging from about 7.0 to about 10.0.

15 If it is desired to include any basic protein such as protamine, it is preferred that the concentration of said basic protein such as protamine in said solution is at least 0.1 µg/ml. The same concentration is preferred for lysozyme or any other substance known to degrade the cell wall of
20 bacteria.

An even more bacteriostatic/fungistatic and/or bacterio-cidal/fungicidal effect was achieved by adding protamine and/or lysozyme to the solution. As less than 0.1 µg/ml
25 protamine and/or lysozyme in a solution comprising citrate and bicarbonate and/or phosphate causes a synergistic bacteriostatic/fungistatic and/or bacteriocidal/fungicidal effect. As a solution comprising citrate, bicarbonate and/or phosphate inhibits the growth of microorganisms and/or fungi
30 or even kills them this capacity is enhanced by adding protamine and/or lysozyme. For example, if it is desired to treat small surfaces the concentration of citrate,

bicarbonate and/or phosphate can be decreased by increasing the concentration of protamine and/or lysozyme without loss of a bacteriostatic/fungistatic and/or bacteriocidal/fungicidal effect. For treatment of larger surfaces the

5   concentration of citrate, bicarbonate and/or phosphate can be increased by decreasing the concentration of protamine and/or lysozyme. This might be desired for economical and/or ecological reasons, because use of protamine or lysozyme in high amounts increases the costs.

10

In a preferred embodiment of the invention the compositions in liquid form (solution) comprise 0.4 mol/l citrate and 0.1 mol/l bicarbonate and 0.1 to 100 µg/ml protamine.

15 In an even more preferred embodiment of the invention the solution comprises 0.4 mol/l citrate and 0.1 mol/l bicarbonate at a pH of 8.0 to 9.5 and 0.1 to 10 µg/ml protamine and/or lysozyme.

20 In a preferred embodiment of the invention the solution comprises 0.2 mol/l citrate and 0.1 mol/l bicarbonate and/or 0.1 mol/l phosphate and 0.1 to 100 µg/ml protamine.

In an even more preferred embodiment of the invention the

25 solution comprises 0.2 mol/l citrate and 0.1 mol/l bicarbonate and/or 0.1 mol/l phosphate at a pH of 8.0 to 10.0 and 0.1 to 10 µg/ml protamine and/or lysozyme.

Embodiments and examples relating to the present invention

30 are now described by way of example only with reference to the following figures.

Figs. 1 and 2 show how plasmids or vectors for use in the method according to the invention have been constructed. A more detailed description is given hereinbelow.

5   Fig. 3 shows the effect of single components (citrate or bicarbonate) of the composition as well as in combination and in the presence of different concentrations of protamine on the growth of *Escherichia coli* DH5α.

10   Fig. 4 shows the effect of single components (citrate or bicarbonate) of the composition at a pH of 8.0 as well as in combination and in the presence of different concentrations of protamine on the growth of *Micrococcus luteus* DSM 348.

15   The initial bacterial inoculum was 10^7 CFU/mL. Results are mean various of three independent experiments. The results show clearly that the combination of the above two components is capable of killing the bacteria tested. In contrast, the single components do not cause the same effect. At a pH of

20   8.0 it is necessary for the composition to additionally comprise protamine to achieve bacterial death. The further addition of lysozyme causes also bacterial death, however, the concentration of protamine needed is lowered to 30 %.

25   Deposition of biological material

Suitable bacterial strains for carrying out the method according to the present invention have been deposited under the Budapest Treaty with the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) in Braunschweig,

30   Germany, under the following Accession Nos.:

Strain	Accession No.
Escherichia coli TAD1	DSM 15050
Pseudomonas fluorescens TAD2	DSM 15051
Rhodopseudomonas palustris TAD3	DSM 15052

5

Examples (section I)

1. Construction of the coding region of the protamine gene

10   Protamine is a peptide which occur in Salmon spermatozoon and
have antimicrobial properties against bacteria and
fungi(1,2,3). Some of the coding sequences and the gene
product are well known (4).

15   To express protamine in bacteria a new gene have to be
constructed because salmon have a different codon usage than
bacteria; for example in salmon 9 of 21 arginies are coded by
AGA and AGG, codons which were very rarely used in E.coli.

20   Therefore, a new gene was constructed under consideration of
the codon usage of *Pseudomonas fluorescens* and *Escherichia
coli* to ensure a optimal synthesis of the protamine peptide
in bacteria. The elected sequence EBLI is shown in SEQ ID NO.
32.

25   The deduced amino acid sequence corresponds to the intact
protamine, e.g. as set forth in SEQ ID NO. 2.

Our strategy was to express the protamine peptide in the
periplasmic space of procaryotes. To realize this strategy
30   the structure gene of EBLI was fused with an appropriate DNA
sequence which codes for a leader peptide that targets the
EBL peptide in the periplasmic space. Therefore, we used the

leader sequence of the pelB gene (5, 6; SEQ ID NO. 34). After translocation of the peptide in the periplasmic space the leader sequence is removed by a signal peptidase.

5   To ensure that the mRNA is efficiently translated a ribosome binding site (AGGAG) was inserted upstream of the coding region. For the sequence and distances between promoter, ribosome binding site and the start codon ATG we utilised the data from the expression vector pET9a (6).

10

To clone the sequence in an expression vector we fused the sequence with the restriction sites for XbaI and BamHI. This whole construct was synthesised (cf. SEQ ID NO. 34).

15   **2. Cloning of the synthesised EBLI construct in the expression vector pET9a**
The expression vector pET9a was constructed for the expression of genes in special *E.coli* strains, which produce T7 polymerase.

20

The XbaI – BamHI EBLI fragment was ligated into the XbaI and BamHI sites of the vector pET9a (6). The ligation was transformed in the *E.coli* strain K12 XL10. Transformants were selected on LB plates containing 30µg/ml kanamycine. After

25   DNA isolation and restriction analysis positive plasmids were sequenced. The plasmid with the correct DNA sequence was named pEBL-FR1 (Figs. 1, 2).

     3. Transformation of the EBLI construct in the *E.coli* strain
30   **BL21 (DE3)**
For expression of the EBLI peptide the plasmid pEBL-FR1 was transformed in the *E.coli* strain BL21(DE3) (Fa. Novagen, 6).

Transformands were selected on LB media containing 30µg/ml kanamycine und 1% glucose. After DNA isolation from growing transformants the plasmids were analyzed by restriction analysis with XbaI and BamHI (data not shown).

5

4. Expression of the EBLI peptide
For the expression of EBLI we used the described *E.coli* strain BL21(DE3) + pEBL-FR1.

10   In this strain the T7 polymerase gene is under control of the lac UV5 promoter and this construct is integrated in the genome (6). Under non induced conditions the lac UV5 promoter is blocked by the lacI repressor. Under induced conditions IPTG inactivates the lac repressor protein and the T7
15   polymerase is expressed. T7 polymerase binds to the T7 promoter region of the plasmid pEBL-FR1 and induces the expression of EBLI.

The expression of the EBLI peptide was induced by adding IPTG
20   to an endconcentration of 0,4mM at a optical density (OD_{600nm}) of 0,5 of the culture. After 2,5 hours additional incubation at 32°C the cells were prepared described in Materials and Methods. A non induced culture serves as control.

25   **5. Localisation and Purification of the EBLI peptide**
From our construction strategy we suspected that EBLI is located at the cell membrane in the periplasmic space. Since the EBLI peptide is highly charged we decided to extract the membrane fraction with H_2O to isolate a enriched fraction of
30   the peptide.

To control the expression and localisation of the EBLI
peptide the following fractions were isolated and analyzed on
a 17% Laemmli SDS page gel (data not shown).

5   **Results:**

Under induction conditions a protein band with a molecular
mass of approximately 16 kDa was detected. This signal was
also detectable in the membrane fraction of induced cells.
Moreover it was extractable with H_2O from the membrane
10   fraction. With micron columns it was possible to concentrate
this protein. In the medium (TCA precipitation) the protein
was not detectable.

The theoretical molecular mass of the intact protamine is
15   about 6 kDa. The difference to the induced peptide could be
explained by the strong positive charge of the protamine
peptide; strong positive charged proteins (protamine,
histones) does not migrate like their molecular mass would be
expected.
20

We see this different migration effect also with commercial
available protamine, which we used as a control. In the
course of isolating EBL I and in order to be able to localize
quickly the target location of the overexpressed EBL I, we
25   decided to raise an antibody against commercial protamine
from Sigma. As a protein chemist, normally we estimate the
homogeneity of the antigen (protamine) before using it to
raise the antibody. Surprisingly, amino acid sequence
analysis revealed many N-terminal amino acids at each step,
30   indicating inhomogenity. By a combination of mass
spectroscopy, HPLC and amino acid sequence analysis, we came
to the definite conclusion that the preparation purchased

from Sigma was composed of at least ten different fragments
of protamine. Unfortunately, we could not identify the
presence of any intact protamine consisting of at least 32
amino acids, indicating that the intact molecule is not
5   present, although our method is extremely sensitive. In this
situation we were not able to use the commercially available
protamine as antigen for raising antibodies, especially since
if this had been homogeneous the higher molecular size of
poly-protamine would have given a better antigenic response.
10

Thus, mass spectroskopy analysis has revealed that this
protamine fraction consists of a mixture of 10 - 15 amino
acids long peptides with a molecular mass of about 2 kDa. In
the SDS page this fraction migrates at a molecular mass of
15   about 10 kDa.

For the verification that we have expressed the intact
protamine, the induced peptide was analysed by mass
spectroskopy.
20

Expression of EBLI in *Pseudomonas fluorescens*
Our aim was the expression of the peptide EBLI in *Pseudomonas
fluorescens* with a special phenotype who tolerate protamine
25   concentrations of more than 40mg/ml in respect to *Escherichia
coli* which does not grow at concentration of 1,5mg/ml.

For expression of the peptide EBLI in *Pseudomonas fluorescens*
we used a system with two plasmids.
30

The first plasmid (pML5T7) contains the T7 polymerase under
the control of the lacUV5 promoter, the lacIq gene and the

gene for tetracyclin resistence. The expression of the T7 polymerase is inducible after adding of IPTG (endconcentration of 2mM , 9).

5 In the second plasmid the structure gene of EBLI is cloned downstream of the T7 promoter.

Therefore we have constructed two plasmids (cf. Figs. 1, 2):
1. pEBL-FR2: The synthesized EBLI construct inclusive the
10   T7 termination signals was cloned under the control of the T7 promoter of the plasmid pBBR1MCS.
2. pEBL-FR4: The plasmid pEBL-FR1 was not replicated in *Pseudomonas fluorescens*. Therefore we fused the plasmid with the replicon pRO1600 from *Pseudomonas aeruginosa*.
15

Construction of the plasmid pEBL-FR2 (Fig. 2)

The vector pBBR1MCS was partially cut with EcoRI and XbaI and then ligated with the fragment EcoRI- XbaI (0,74 kB) isolated
20 from the plasmid pEBL-FR1. This EcoRI-XbaI fragment contains the synthesized EBLI construct and the T7 termination signals.

The vector pBBR1MCS was partially cut with EcoRI and XbaI and
25 then ligated with the fragment EcoRI- XbaI (0,74 kB) isolated from the plasmid pEBL-FR1.

From growing transformants the plasmid was isolated and restricted with XbaI and EcoRI. The resulting fragment has a
30 size of 0,74 kB and was not distinguishable from an other fragment resulting from the second EcoRI site of the vector. Therefore we restricted the plasmid with XbaI and NcoI

resulting in sigals of 0,5kB for the vector and 1,23kb for the correct construct.

Transformation of the strain wis 437 from *Pseudomonas*
5   ***fluorescens***

In a first transformation the strain 437 was transformed by electroporation with the plasmid pML5T7 (11). For the selection of transformants the cells were plated on LB plates with a tetracyclin concentration of 10µg/ml. After DNA
10   isolation the plasmid was analyzed by restrictions analysis.

In a second transformation the strain wis 437 + pML5T7 was transformed by electroporation with the plasmid pEBL-FR2 and with the vector pBBR1MCS. For the selection of transformants
15   the cells were plated on LB plates with a endconcentration of 10µg/ml tetracycline, 340µg/ml chloramphenicol and 1% glucose. From growing transformants plasmids were isolated and controlled by a restriction analysis (data not shown).

20   **Expression of EBLI in *Pseudomonas fluorescens***
The expression of the EBLI peptide was induced by adding IPTG to an endconcentration of 2mM at a optical density (OD_{600nm}) of 0,5 of the culture. After 2,5, 5 and 7,5 hours additional incubation at 32°C the cells were broken with glass beads as
25   described in Materials and Methods. A non induced culture serves as an control. To reduce the stress for the growing cells (the media contain tetracycline and kanamycine and the cells express EBLI) we have decreased the antibiotica concentration for tetracycline to 5µg/ml and for kanamycine
30   to 10µg/ml. The grow curve showed that under low antibiotic conditions the cells have a slight growth advantage without loss of plasmids (Data not shown).

Induction of the EBLI peptide in *P.fluorescens* with IPTG

The cultures were splitted. To one culture IPTG was added to induce the expression of the EBLI peptide. The other culture
5   served as an uninduced control. After additional incubation of 2,5, 5 and 7,5 hours at 32°C the cells were harvested, broken with glass beads and the total cell protein was isolated (see materials and methods). The proteins were analyzed on a 17% Laemmly SDS page gel (data not shown).
10

Results:

Under induction conditions an expressed peptide band with a molecular mass of approximately 16 kDa was detected. After 7,5 hours additional incubation under induction conditions
15   the highest concentration of the induced protein was yielded. Under low antibiotic concentrations the gel showed a slightly more expression of the peptide caused by the better growth of the cells.

20   **Construction of the plasmids pEBL-FR4 (Fig. 1)**

The plasmid pEB1 (10) was restricted with the restriction enzyme PstI and the resulting fragment (replikcon pRO1600: 1,8kB) was cloned in the vector pUC19 (restricted with Pst). After plasmid isolation from growing transformants the
25   pRO1600 fragment was isolated with the restriction enzymes PaeI and SalI. This fragment was cloned in the plasmid pEBL-FR1 (restricted with PaeI (SphI) and SalI)(see Fig. 1).

30

Materials and Methods

1. Organisms and Plasmids

For the experiments certain *Pseudomonas fluorescens* and *Pseudomonas aeruginosa* strains were used as well as different *Escherichia coli* strains. Table 1 shows the utilized strains and their characteristics.

Table 1: *Pseudomonas* and *Escherichia coli* strains

Strain	Relevant genotypes
E.coli	
K12 XL10	$Tet^R\Delta$(mcrA) 183 Δ(mcrCB-hsdSMR-mrr) 173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte[F'proAB lacI^{q}ZΔM15 Tn10 (Tet^R) Amy Cam^R]
BL21(DE3)	F^- ompT hsdS$_B$ (r_B^- m_B^-) gal dcm (DE3)
DH5 alpha	*supE44 ΔlacU169 (φ80lacZ ΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*
Pseudomonas fluorescens	
wis 437 (DSM50106)	wild type
wis 437 + pML5T7	pML5T7
wis 437 + pML5T7 + pEBL-FR2	pEBL-FR2

Table 2: Plasmids

Plasmid	Relevant genotypes [a]	host strain
pET9a	km^r, T7 promoter upstream of the cloning sites *Bam*HI and *Xba*I	*E. coli* BL21(DE3)
pBBR1MCS	cm^r, T7 promoter upstream of a polycloning site	*E. coli* DH5 alpha
pEB1	tet^r, the T7 polymerase under control of the lacUV5 promoter, lacIq gene, origin of replication (pRO1600) from *Pseudomonas aeruginosa*	*E. coli* DH5 alpha
pML5T7	tet^r, the T7 polymerase under control of the lacUV5 promoter and the lacIq gene	*E. coli* DH5 alpha
pUC19	amp^r, polycloning site	*E. coli* DH5 alpha
pEBL-FR1	synthesized sequence of EBLI	*E. coli* BL21(DE3)
pEBL-FR2	synthesized sequence of EBLI	*E. coli* DH5 alpha
pEBL-FR4	synthesized sequence of EBLI	*E. coli* DH5 alpha
pEBL-FR3	replicon pRO1600 from *Pseudomonas aeruginosa*	*E. coli* DH5 alpha

a: km: kanamycin, cm: chloramphenicol, tet: tetracyclin, amp: ampicilline, r: resistent

Chemicals and enzymes used

Product	Source
40% acrylamide- bisacrylamide soluton (29:1) (Rotiphorese ®)	Fa Roth (Karlsruhe, Germany)
agar and media	Fluka Chemie GmbH (Buchs, Germany)
Agarose, Seakem LE	FMC Bioproducts Rockland, Maine USA
Ammoniumperoxodisulfate (APS)	Fa Roth (Karlsruhe, Germany)
Ampicilline (Amp)	Sigma Aldrich (Taufkirchen, Germany)
Boric acid	Fa Roth (Karlsruhe, Germany)
Bromphenol blue	Sigma Aldrich (Taufkirchen, Germany)
Chloramphenicole (Camp)	Fa Roth (Karlsruhe, Germany)
Coomassie brillant blue R250	Fa Roth (Karlsruhe, Germany)
DNA Isolation kits	Fa Qiagen, (Hilden, Germany)
Dithiothreitol (DTT)	Fa Roth (Karlsruhe, Germany)
Ethanol	Fa Roth (Karlsruhe, Germany)
Ethylenediamine tetraacetic acid (EDTA)	Fa Roth (Karlsruhe, Germany)
Glacial acetic acid	Fa Roth (Karlsruhe, Germany)

Glycerol	Fa Roth (Karlsruhe, Germany)
Glycine	Fa Roth (Karlsruhe, Germany)
IPTG	Fa Roth (Karlsruhe, Germany)
Kanamycine (Kan)	Sigma Aldrich (Taufkirchen, Germany)
Methanol	Fa Roth (Karlsruhe, Germany)
Potassium dihydrogen phosphate	Fa Roth (Karlsruhe, Germany)
Potassium chloride	Fa Roth (Karlsruhe, Germany)
Protamine from salmon	Sigma Aldrich (Taufkirchen, Germany)
Protein marker (low range)	Sigma Aldrich (Taufkirchen, Germany)
Restrictions enzymes, T4 ligase, DNA marker	MBI Fermentas (St. Leon-Rot, Germany
Sodium chloride	Merck (Darmstadt, Germany)
Sodium dihydrogen phosphate monohygrate	Merck (Darmstadt, Germany)
Sodium dodecyl sulfate (SDS)	Fa Roth (Karlsruhe, Germany)
Tetracycline (Tet)	Sigma Aldrich (Taufkirchen, Germany)
N,N,N´,N´- Tetramethylethylendiamine (TEMED)	Fa Roth (Karlsruhe, Germany)
Tris base	Fa Roth (Karlsruhe, Germany)
Xylene xyanol	Fa Roth (Karlsruhe, Germany)

Media: All strains were grown on rich medium (Luria-Bertani (LB)) containing 1% glucose at 32°C. LB media and plates were

5   purchased from Fa. Fluka Chemie GmbH (Buchs, Germany):

LB media:

Tryptone   10g/l

Yeast extract   5g/l

Sodium chloride   5g/l

10 Dissolve 20g in 1 litre destilled water and adjust the pH to 7,2. Sterilize by autoclaving at 121 °C for 15 min.

LB agar:

Tryptone   10g/l

Yeast extract   5g/l

15 Sodium chloride   5g/l

Agar   10g/l

Dissolve 30g in 1 litre destilled water and adjust the pH to 7,2. Sterilize by autoclaving at 121 °C for 15 min.

20 For selection of transformed *E.coli* we used the following antibiotic concentrations:

Tetracycline 10µg/ml

Kanamycine 10µg/ml

Chloramphenicol: 25µg/ml

25 Ampicilline: 40µg/ml

For selection of transformed *Pseudomonas fluorescens* we used the following antibiotic concentrations:

Tetracycline 10µg/ml

Kanamycine 30µg/ml

30 Chloramphenicol: 340µg/ml

Buffers

TBE Puffer: per 1 litre

5,4g Tris base

2,75g boric acid

5   20ml 0,5M EDTA pH8,0

5x agarose gel loading buffer:

0,25% bromphenol blue

0,25% xylene cyanol FF

10   30% glycerol

Phosphat-buffered saline (PBS)

8g NaCl

0,2g KCl

15   1,44gNa$_2$HPO$_4$

0,24g KH$_2$PO$_4$

adjust pH to7,4 with HCl and autoclave for 20 min at 121°C

Plasmid constructions:

20   All plasmids were constructed in E.coli DH5 alpha by standard

protocols

Protocol for competent cells of E. coli

1. Pick a single colony (2-3 mm in diameter) and inoculate
   a 5ml culture of LB broth. Incubate the culture
25   overnight with at 37°C with vigorous shaking.
2. Inoculate 100ml LB broth with 1ml of the over night
   culture and incubate at 37°C.
3. When a optical density (a OD$_{600}$) of 0,3-0,4 is reached
   the cells were transferred in sterile centrifugation
30   tubes and stored for 10 min on ice
4. Recover the cells by centrifugation at 4000 rpm for 10
   min at 4°C in a Sorvall SS34 rotor.

5. Decant the media from the cell pellets. Stand the tubes in an inverted position for 1 min to allow the last traces of media to drain away.

6. Resuspend each pellet in 50 ml of ice-cold 0.1 M $CaCl_2$ and store on ice for 20min.

7. Recover the cells by centrifugation at 4000 rpm for 10 min at 4°C in a Sorvall SS34 rotor.

8. Decant the fluid from the cell pellets and stand the tubes in an inverted position for 1 min to allow the last traces of fluid to drain away.

9. The pellet was resuspended in 9 ml of ice-cold 0.1 M $CaCl_2$.

10. Ice cold glycerol was added to the cell suspension to a end concentration of 15%

11. The cell suspension was portionated (500µl in sterile microfuge tubes) and stored at -80°C

Transformation of competent cells

1. To 200µl competent cells 20µl of the ligation mixture (not more than 200ng DNA) was added and the tube stored on ice for 40min.

2. heat shock at 42°C for 2min

3. Rapidly transfer the tubes to an ice bath. Allow the cells to chill for 1-2 min.

4. 1ml of LB medium was added and the cells than incubated for 1hour at 37°C

5. The cells were harvested in a microfuge (4000rpm for 5min) and spread on a agar plate supplemented with the appropriate antibiotics.

6. Colonies should appear in 12-16 hours.

Mini Preparation of plasmid DNA

1. Resuspend pelleted bacterial cells in 250 µl Buffer P1 and transfer to a microcentrifuge tube.

2. Add 250 µl Buffer P2 and gently invert the tube 4-6 times to mix.

3. Add 350 µl Buffer N3 and invert the tube immediately but gently 4-6 times.

4. Centrifuge for 10 min at maximum speed in a tabletop microcentrifuge.

5. Apply the supernatants form step 4 to the QIAprep column by decanting or pipetting.

6. Centrifuge for 30-60 s. Discard the flowthrough.

7. Wash the QIAprep spin column by adding 0.5 ml Buffer PB and centrifuging for 30-60 s. Discard the flowthrough.

8. Wash QIAprep spin column by adding 0.75 ml Buffer PE and centrifuging for 30-60 s.

9. Discard the flowthrough and centrifuge for an additional 1 min to remove residual wash buffer.

10.   Place the QIAprep column in a clean 1.5 ml microcentrifuge tube. To elute DNA, add 50 µl Buffer EB (10 mM Tris-Cl, pH 8.5) to enter the center of each QIAprep column, let stand for 1 min and centrifuge for 1 min.

11.   For a restriction analysis we used 3µl for high copy plasmids and 8µl for low copy plasmids

Midi Preparation of plasmid DNA

1. Pick a single colony from a freshly selective plate and inoculate a starter culture of 2-5 ml LB medium containing the appropriate selective antibiotic. Incubate for ~8 h at 37°C with vigorous shaking (~300 rpm).

2. Dilute the starter culture 1/500 to 1/1000 into selective LB medium. For high-copy plasmids inoculate 25 ml medium. For low-copy plasmids, inoculate 100 ml medium. Grow at 37°C for 2-16 h with vigorous shaking (~300 rpm).

3. Harvest the bacterial cells by centrifugation at 4000 rpm for 15 min at 4°C in a Sorvall SS34 Rotor .

4. Resuspend the bacterial pellet in 4 ml of Buffer P1.

5. Add 4 ml of Buffer P2, mix gently but thoroughly by inverting 4-6 times, and incubate at room temperature for 5 min.

6. Add 4 ml of chilled Buffer P3, mix immediately but gently by inverting 4-6 times and incubate on ice for 15 min.

7. Centrifuge at 8000 rpm for 30 min at 4°C in a Sorvall SS34 Rotor. Remove supernatant containing plasmid DNA promptly.

8. Equilibrate a QUIAGEN-tip 100 by applying 4 ml Buffer QBT and allow the column to empty by gravity flow.

9. Apply the supernatant from step 7 to the QUIAGEN-tip and allow it to enter the resin by gravity flow.

10.   Wash the QUIAGEN-tip with 2 x 10 ml Buffer QC.

11.   Elute DNA with 5 ml Buffer QF.

12.   Precipitate DNA by adding 3.5 ml room temperature isopropanol to the eluted DNA. Mix and centrifuge immediately at 8000 rpm for 30 min at 4°C in a Sorvall SS34 Rotor. Carefully decant the supernatant.

13.   Wash DNA pellet with 2 ml of room-temperature 70% ethanol and centrifuge at 8000 rpm for 10 min in a Sorvall SS34 rotor. Carefully decant the supernatant without disturbing the pellet.

14.   Air-dry the pellet for 5-10 min and redissolve the
DNA in a suitable volume of H_2O

Restriction analysis, DNA ligations

5   Enzymatic reactions were performed according the manuals of
MBI Fermentas.

Standard restriction analysis were performed in volume of
20µl with 5Unit of the appropriate enzyme.

10

Standard agarose gels: 0,7% , 40ml
0,28g agarose were dissolved in 40ml TBE buffer. The agarose
solution was melted in a micro wave until the solution is
clear. After cooling to 50°C 4µl of a 1% Ethidium bromid

15   solution was added.

After the gel is completely set the samples of DNA were mixed
with 5x gel loading buffer and load into the slots.The gel
was running at 100 Volt in a gel chamber from Stratagene.

20

DNA fragment isolation from Agarose Gels
   1. Excise the DNA band from the agarose gel with a clean,
       sharp scalpel.
   2. Weigh the gel slice. Add 3 volumes of Buffer QX1 to 1
25   volume of gel for DNA fragments of 100 bp to 4 kb.
   3. Resuspend QIAEX II by vortexing for 30 sec.
   4. Add QIAEX II according to the table below and mix.
       Incubate at 50°C for 10 min. Mix every 2 min to keep
       QIAEX II in suspension.
30   5. Centrifuge the sample for 30 sec and carefully remove
       supernatant with a pipet.
   6. Wash the pellet with 500 µl of Buffer QX1.

7. Wash the pellet twice with 500 µl of Buffer PE.

8. Air-dry the pellet for 10-15 min or until the pellet becomes white.

9. To elute DNA, add 20 ml of 10 mM Tris-HCl, pH 8.5 or H_2O and resuspend the pellet by vortexing. Incubate at room temp. for 5 min.

10.   Centrifuge for 30 sec. Carefully pipet the supernatant into a clean tube.

11.   Optional: repeat steps 9 and 10 and combine the eluates.

Electroporation von *Pseudomonas fluorescens*

The electroporation conditions are described by Bloemberg et .al. (11)

- Prepare an 2ml sample of an overnight culture of cells in LB media and inoculate into 50ml of fresh LB

- incubate at 32°C with shaking until the late log phase

- When an optical density (OD $_{600nm}$) of 0,5 - 1,0 is reached (10^{10}Cell/ml) chill the culture for 30min on ice

- centrifuge the culture at 4°C in a SS34 rotor for 10min at 4000rpm

- wash the cell with 25 ml ice-cold 10 % glycerol

- wash the cells with 1ml ice-cold 10 % glycerol

- resuspend the cells in 200µl ice-cold 10 % glycerol

- load a pre chilled 2-mm-gap electroporation cuvette with 50µl of the cellsuspension

- add 1-3µl DNA (0,5- 1µg DNA)

- electroporation was performed a 1,8kV with an E.coliPulser (Bio-Rad, Melville,N.Y.)

- after the pulse resuspend the cells in 1ml LB media and incubate for 1hour at 32°C

- After electroporation the cells were incubated for one hour at 32°C, centrifuged for 5min at 4000rpm and than plated on LB agar with 1% glucose, supplemented with the appropriate antibiotics:

5

Expression and purification of the EBLI Peptid in E.coli strain BL21 (DE3)

1. Expression of the EBLI Peptid

The expression of the target gene followed the protocols of

10 Fa Novagen (6).

- Inoculate a 5ml LB culture containing 30µg/ml kanamycin and 1% glucose. Incubation at 32°C over night under vigorous shaking.

- Inoculate a 100ml LB Medium culture containing 30µg/ml

15 Kanamycin, 1% Glukose

with 1ml of the over night culture.

- Inkubation at 32°C under under vigorous shaking until a optical density (OD $_{600nm}$)of 0,5 is reached

- split the culture into 2x 50ml cultures:

20   o A: uninduced control

        o B: IPTG was added to a final concentration of 0,4mM (induced culture)

The incubation was continued for 2,5 hours

- The cells were harvested by centrifugation at 4000 rpm

25   for 10 min at 4°C in a Sorvall SS34 rotor.

- Traces of media from the pellet were removed with a pipette. The pellet was than suspended in 500µl phosphate buffered saline (PBS) and stored at -20°C.

- The supernatant was stored in -20°C.

30

2. Preparation of the medium

The trichloroacetic acid (TCA) preparation followed the protocol of Fa Novagen (6)

5   - 100µl (1/10 volume) of 100% TCA to 1ml of medium was added and vortex for 15 sec.

- Incubation on ice for a minimum of 15min.

- The suspension was centrifuged at 12000 rpm for 10 min at 4°C in a Sorvall SS34 rotor.

- The pellet was washed with 1ml acetone and centrifuged

10   at 12000 rpm for 5 min at 4°C in a Sorvall SS34 rotor

- This step was repeated.

- The pellet was air dried for about 60min and than resuspended in 100µl PBS.

- 100µl 2x SDS gel loading buffer was added

15   - For denaturation of the proteins the probe was heated for 3min at 90°C and stored at -20°C.

3. Disruption of the cells

The cells were mechanically disrupted by vortexing with

20 glassbeads (25µm).

- The cell suspension (in 500µl PBS) was transferred in a reaction tube. Glass beads were added until the lower meniskus of the suspension is reached.

- The suspension was vortexed for 20sec and than stored on

25   ice for 20sec. This step was 12x repeated (whole vortexing time of 4min).

- The glass beads were washed with 500µl of PBS. This step was repeated until the endvolume of the wash fraction was 1ml.

30   - To 20µl of this suspension 20µl 2x SDS gel loading buffer was added

- For denaturation of the proteins the probe was heated for 3min at 90°C and stored at -20°C

4. Präparation of the cellwall- membranefraction.

5   To isolate the cellwall- membranefraction the disrupted cells were centrifuged at 12000 rpm for 10 min at 4°C in a Sorvall SS34 Rotor

o supernatant: cytoplasmic fraction (about 800µl): To 20µl of the fraction 20µl 2x SDS gel loading buffer was added, heated 3min at 90°C and then stored at -20°C

o pellet: cellwall- membrane fraction (suspended in 100µl PBS): To 12,5µl of the fraction 12,5µl 1 2x SDS gel loading buffer was added, heated 3min at 90°C and then stored at -20°C

o

5. Isolation of the peptide EBLI from the water soluble fraction of the cellwall- membrane fraction

20   The pellet (cellwall- membrane fraction) was extracted with 500µl H₂O and centrifuged at 12000 rpm for 5 min at 4°C in a Sorvall SS34 Rotor.

For quantitative extraction this step was repeated for three 25   times and the supernatants were combined.

o pellet: water insoluble membranefraction (suspended in 100µl PBS): To 10µl of the fraction 10µl 2x SDS gel loading buffer was added, heated 3min at 90°C and then stored at -20°C

o supernatant: water soluble membrane fraction (about 1400 µl): To 25µl of the fraction 25µl 2x SDS gel

loading buffer was added, heated 3min at 90°C and then stored at
-20°C

5 **6. Concentration of the water soluble membrane fraction**

1400µl were concentrated on a Micron YM-3 column to an end volume of 160µl. To 20µl of the fraction 20µl 2x SDS gel loading buffer was added, heated 3min at 90°C and then stored at -20°C.

10

Concentrating of protein solutions

To concentrate the water soluble membrane fraction we use the Microcon Centrifugal Filter Devices YM-3 (Millipore, Bedford, USA).

15   1. Insert Microcon sample reservoir into vial
       2. Pipette solution into sample reservoir· (0,5ml maximum volume), without touching the membrane with the pipette tip. Seal with attached cap.
       3. place the tube in a centrifuge for 1,5ml tubes
20   4. centrifuge at 13000g for 30min (400µl are spin through the columm)
       5. if nescessary add additional 400µl on the column and repeat the centrifugation.
       6. remove assembly from centrifuge. Seperate vial from the
25   sample reservoir.
       7. Place sample reservoir upside down in a new vial, then spin 3 minutes at 1000xg to transfer concentrate to vial.
       8. Remove from centrifuge. Seperate sample reservoir. Store
30   the concentrate at -20°C.

Literature:

1. **Hirsch, J.G. (1958)** Bacteriacidal action of histone. *J.Exp.Med.* **107**: 925-944

2. **Islam, N.M., Itakura,T., Motohiro,T. (1984)** Antibacterial spectra and minimum inhibitory concentration of clupeine and salmine. *Bull. Jpn. Soc. Sci. Fish* **50**: 1705-1708

3. **Johansen,C., Gill,T., Gram,L. (1995)** Antibacterial effect of protamine assayed by impedimetry. *J. Appl. Bacteriol.* **78**: 297-303

4. **Ando ,T., Watanabe, S. (1969)** A new method for fractionation of protamines and the amino acid sequences of samline and three components of iridine. *Int. J. Protein Res.* **1**: 221-224

5. **Wickner,W., Driessen, A.J.M., Hartl, F.-U. (1991)** *Annu.Rev. Biochem.* **60** 101-124

6. **Produktbeschreibung der Fa Novagen. (1994)** The pET System: Your choice for expression. *inNovations* **1**: 1. 1- 58

7. **Sambrook ,J., Fritsch, E.F., Maniatis,T. (1989)** Molecular cloning: a laboratory manual, 2nd ed. *Cold Spring Harbor Laboratory Press*, **Cold Spring Harbor, N.Y.**

8. **Laemmli U.K. (1970)** Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227** 680- 685

9. **Rosenau, F., Liebeton, K., Jaeger, K.-E. (1998)** Überexpression extrazellulärer Enzyme in *Pseudomonas aeruginosa Biospektrum* **4**: 38-41

10.   Brunschwig, E., Darzins, A., (1992) A two-component
       T7 system for the overexpression of the genes in
       Pseudomonas aeruginosa. Gene **111**: 35- 41

11.   Bloemberg, G.V., O'Toole, G.A., Lugtenberg, B.J.J.,
       Kolter, R. (1997) Green fluorescent protein as a marker
       for *Pseudomonas* spp. *Appl. and Envir. Microbiol.* **63**: 11,
       4543- 4551

12.   Kovach, M.E., Phillips, R.W., Elzer, P.H., RoopII,
       R,M., Peterson, K.M. (1994) pBBR1MCS: A broad-host-range
       cloning vector *BioTechniques* **16**: 5, 801-802

13.   Hanahan, D. (1983) Studies on transformation of
       Escherichia coli with plasmids.   *J. Mol. Biol.* **166**:
       557-580

14.   DSMZ- Deutsche Stammsammlung von Mikroorganismen
       und Zellkulturen GmbH, Braunschweig, Germany

15.   Yanisch-Perron, C., Vieira, J. and Messing, J.
       (1985) *Gene* 33, 103-119

Examples (section II)

Bacterial growth conditions

Bacteria are cultured in Luria-Bertani broth (LB, WWW 2001)
respectively tryptic soy broth (TSB, DIN EN 1040, 1997) at
37°C. All bacterial strains are maintained as frozen stocks
at - 80°C in TSB, Luria-Bertani broth.

Throughout the experiments strains are subcultured every 2
weeks on TSA (tryptic soy agar; 15 g l^{-1} agar) or LBA (Luria-
Bertani agar; 15 g l^{-1} agar) depending upon the type of
bacteria and experiment and kept at 4 °C. The incubation
temperature is 37°C except for photo-heterotrophic bacteria

(30 °C). Before experimental use, cultures are propagated twice in the corresponding broth (pH 7.2 ± 0.2) for 18-24h.

Parameters applied to determine the inhibitory effect of Protamine

The growth curve is determined by measuring the optical density (OD), growth rate and doubling time. Additionally, the number of colony forming units is determined by counting bacterial spots.

Bacterial growth is monitored by recording the increase in optical density (OD) at 560 nm in a spectrophotometer (Ultrospec 1000, Pharmacia Biotech, England). Sterile medium is used as reference. Growth rate (μ) and doubling time (t_d) are calculated during the logarithmic growth phase according to SCHLEGEL (1992). At given times, 100 µl aliquots of the samples are removed and spread on corresponding agar plates. If necessary, the samples are diluted by factors between 10^2 – 10^8 to ensure good count ability. Colony forming unit determination (CFU/ml) is carried out in duplicate using a CCD camera module (Sony Corp., Japan) applying the BioProfil Software V 2000.01 (Vilber Lourmat, France).

Use of the basic bactericidal activity test method DIN EN 1040 (DEV 1997)

Basic bactericidal activity is determined according to the European standard method DIN EN 1040 (DEV, 1997). Bacterial cells are cultured on tryptone soy agar plates (tryptone-soy-agar, TSA, tryptone: 15.0 $g*l^{-1}$; soy-peptone: 5 $g*l^{-1}$; NaCl: 5 $g*l^1$; agar: 15 $g*l^{-1}$). Bacterial suspensions of 1.5 * 10^8 – 5.0 * 10^8 bacteria per milliliter are subjected to several concentrations of Protamine. After an incubation time of 60

minutes, two 100 µl aliquots of the samples are filtered
separately through presterilized cellulosic membrane filters
(Osmonics, Minnetonka, USA, diameter 47 mm; pore size 0.45
µm; Catalog No. E04WG047S1). The filters are firmly placed
5 onto TSA-plates and incubated at 37 °C for 24 – 48 hours. The
number of colony forming units is determined by counting
bacterial spots.

Reagents

10 A commercially available protamine from Salmon is obtained
from Sigma Aldrich (Taufkirchen, Germany). A stock solution
is prepared by dissolving protamine in distilled water. The
solution is then sterilized with Rotabilo® filter units (0.22
µm; Carl Roth, Karlsruhe, Germany) and stored at –20 °C until
15 use.

**Determination of bactericidal effect of biological buffers
with or without protamine on gram-negative bacteria**
In all experiments the starting inoculum is 10^7 cells. The
20 data shown are average means of three replicates.

Table 3 : Effect of biological buffers with or without
different concentrations of Protamine on the growth of
Escherichia coli DH5α

- 25 -

	control	0.1 M NaHCO₃	0.4 M citrate	0.1 M NaHCO₃ + 0.4 M citrate
µ [h⁻¹]	0,468	0,230	0,185	0
t_d [h]	1,48	3,01	3,75	0

0.1 M NaHCO₃ + 0.4 M citrate Protamine [mg/ml]	0.005	0.01	0.02	0.04	0.08
μ [h⁻¹]	0	0	0	0	0
t_d [h]	0	0	0	0	0

The results are shown in Fig. 3.

Determination of bactericidal effect of biological buffers with or without protamine on gram-positive bacteria

In all experiments the starting inoculum is 10^7 cells. The data shown are average means of three replicates.

Table 4: Effect of biological buffers with or without different concentrations of Protamine on the growth of *Micrococcus luteus* DSM 348

	control	0.1 M NaHCO₃	0.4 M citrate	0.1 M NaHCO₃ + 0.4 M citrate
μ [h⁻¹]	0,389	0	0	0
t_d [h]	1,78	0	0	0

0.1 M NaHCO₃ + 0.4 M citrate protamine [mg/ml]	0.005	0.01	0.02	0.04	0.08	0.16	0.32	0.64	1.28
μ [h⁻¹]	0	0	0	0	0	0	0	0	0
t_d [h]	0	0	0	0	0	0	0	0	0

The results are shown in Fig. 4.

Further Literature:

Hirsch, J.G. (1958) Bacteriacidal action of histone. *J.Exp.Med.* **107**: 925-944

Islam, N.M., Itakura,T., Motohiro,T. (1984) Antibacterial spectra and minimum inhibitory concentration of clupeine and salmine. *Bull. Jpn. Soc. Sci. Fish* **50**: 1705-1708

Johansen,C., Gill,T., Gram,L. (1995) Antibacterial effect of protamine assayed by impedimetry. *J. Appl. Bacteriol.* **78**: 297-303

Ando ,T., Watanabe, S. (1969) A new method for fractionation of protamines and the amino acid sequences of samline and three components of iridine. *Int. J. Protein Res.* **1**: 221-224

Wickner,W., Driessen, A.J.M., Hartl, F.-U. (1991) *Annu.Rev. Biochem.* **60** 101-124

Produktbeschreibung der Fa Novagen. (1994) The pET System: Your choice for expression. *inNovations* **1**: 1. 1- 58

Sambrook ,J., Fritsch, E.F., Maniatis,T. (1989) Molecular cloning: a laboratory manual, 2nd ed. *Cold Spring Harbor Laboratory Press*, Cold Spring Harbor, N.Y.

Laemmli U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227** 680- 685

Rosenau, F., Liebeton, K., Jaeger, K.-E. (1998) Überexpression extrazellulärer Enzyme in *Pseudomonas aeruginosa Biospektrum* **4**: 38-41

Brunschwig, E., Darzins, A., (1992) A two-component T7 system for the overexpression of the genes in *Pseudomonas aeruginosa. Gene* **111**: 35- 41

Bloemberg, G.V., O´Toole, G.A., Lugtenberg, B.J.J., Kolter, R. (1997) Green fluorescent protein as a marker

for *Pseudomonas* spp. *Appl. and Envir. Microbiol.* **63**: 11, 4543- 4551

Kovach, M.E., Phillips, R.W., Elzer, P.H., RoopII, R,M., Peterson, K.M. (1994) pBBR1MCS: A broad-host-range cloning vector *BioTechniques* **16**: 5, 801-802

Hanahan, D. (1983) Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* **166**: 557-580

DSMZ- Deutsche Stammsammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany

Yanisch-Perron, C., Vieira, J. and Messing, J. (1985) *Gene* **33**, 103-119

Ando, T., Yamasaki, M., Suzuki, K. (1973)
Protamines: isolation, characterization, structure and function, p 1-109 *In*: A. Kleinzeller and H:G. Wittmann (ed), Molecular biology, biochemistry and biophysics. Chapman & Hall Ltd., London, United Kingdom

Blackburn, P., Polak, J., Gusik, S. A., Rubing, S. D (1989)
Nisin composition for use as enhanced broad range bactericides. International Patent Application publication WO 89112399.

Christensen, B., Fink, J., Merrifield, R.B., Mauzerall, D. (1988)
Channel-forming properties of cecropins and related model compounds incorporated
into planar lipid membranes. *Proc. Natl. Acad. Sci. USA* **85**:5072-5076

Hanahan, D. (1983)

Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* 166: 557-580

5   Hirsch, J. G. (1958)
Bactericidal action of histones. *J. Exp. Med.* **108**:925-944

Islam, N.M., Itakura, T., Motohiro, T. (1984)
Antibacterial spectra and minimum inhibitory concentration

10   of clupeine and salmine. *Bull. Jpn. Soc. Sci. Fish.* **50**:
1705-1708

Jack, R.W., Tagg, J.R., Ray, B. (1995)
Bacteriocins of gram-positive bacteria. *Microbiol. Rev.* 59

15   (2): 171-200

Johansen, C., Gill, T., Gram, L. (1996)
Changes in cell morphology of *Listeria monocytogenes* and
Shewanella putrefaciens by the action of protamine. *Appl.*

20   *Environ. Microbiol.* **62**:1058-1064

JOHANSEN, C., GILL, T., GRAM, L. **(1995)**
Antibacterial effect of protamine assayed by impedimetry.
J. Appl. Bacteriol. **78**: 297-303

25   JOHANSEN, C., VERHEUL, A., GRAM, L., GILL, T., ABEE, T. **(1997)**
Protamine-induced permeabilization of cell envelopes of
gram-positive and gram-negative bacteria. *Appl. Environ.*
Microbiol. 63**(3)**:1155-1159.

30

KAMAL, M., MOTOHIRO, T. ITAKURA, T. (1986)

Inhibitory effect of salmine sulfate on the growth of molds. *Bull. Jpn. Soc. Sci. Fich.* **52**: 1061-1064.

5   **KAGAN, B.L., SELSTED, M.E., GANZ, T., LEHRER, R.I. (1990)**

Antimicrobial defensin peptides form voltage-dependent ion-permeable channels in planar

lipid bilayer membranes. *Proc. Natl. Acad. Sci. USA* **87**:210-214

10

Kagan, B.L., Selsted, M.E., Ganz, T., Lehrer, R.I. (1990) Antimicrobial defensin peptides form voltage-dependent ion-permeable channels in planar lipid bilayer membranes. *Proc. Natl. Acad. Sci. USA* **87**:210-214

15

Kalchayanand, N., Hanilin, M.B., Ray, B. (1992) Sublethal injury makes Gram-negative and resistant Gram-positive bacteria sensitive to the bacteriocins, pediocin AcH and nisin. *Lett. Appl. Microbiol.* **15**:239-243.

20   Louie, A.J., Dixon, C.H. (1974)

Enzymatic modifications of the protamines. II. Separation and characterization of phosphorylated species of protamines from trout testis. *Can. J. Biochem.* **52**: 536-546

25

Mulholland, B., Mellersh, A.R .(1987) The antibacterial activity of protamine and polybrene. *J. Hosp. Infect.* **10**:305-307

30   Nes, I.F., Holo, H. (2000)

Class II antimicrobial peptides from lactic acid bacteria. *Biopolymers* (Peptide Science) **55**:50-61.

O'Brien, T.F., Eskildsen, M.A., Stelling, J.M. (2000)
The complex processes of antimicrobial resistance and the
information needed to manage them. *Mil Med.* 165 (7 Suppl
2):12-15

Rubinstein, E. (1999)
Antimicrobial resistance — Pharmacological solutions.
Infection. Suppl 2:S32-S34.

Stevens, K. A., Sheldon, B.W., Klapes, N.A., Klaenhammer,
T. R. (1991)
Nisin treatment for inactivation of *Salmonella* species and
other gram-negative bacteria. *Appl. Environ. Microbiol.*
57:3613-3615.

Vaara, M. (1992)
Agents that increase the permeability of the outer
membrane. *Microbiol. Rev* 56**(3)**:395-411.

Vaara, M., Vaara, T. (1983)
Polycations as outer membrane-disorganizing agents.
Antimicrob. Agents Chemother. **24**:114-122

Yanagimoto, T., Tanaka, M., Nagashima, Y. (1992)
Changes in the antibacterial activity of salmine during
its maillard reaction. *Bull. Jpn. Soc. Sci.*
Fish. **58**:2153-2158

Al-Sharidah, A., Richardt, A., Golecki, J.R., Dierstein,
R., Tadros, M.H. (2000)

Isolation and characterization of two hydrocarbon-
degrading *Bacillus subtilis* strains from oil contaminated
soil of Kuwait. Microbiol. Res. **155**:157-164.

5   DEV (1997)

Chemical disinfectants and antiseptics; Basic bactericidal
activity – Test method and requirements (phase 1): German
version EN 1040:1997.

10   SCHLEGEL, H.G. (1992)

Allgemeine Mikrobiologie. 7th revised edition. Thieme,
Stuttgart, New York.
MILLER, J.H. (1972)

15   EXPERIMENMTS IN MOLECULAR GENETICS. COLD SPRING HARBOUR, NY. COLD
SPRING HARBOUR LABORATORY.

NAVARRO, L., ZARAZAGA, M., SÁENZ, F., RUIZ-LARREA, F., TORRES,
C. (2000)
20   Bacteriocin production by lactic acid bacteria isolated
from Rioja red wines. J. Appl. Microbiol. 88:44-51

SAKAI, M., FUJI-KURIYAMA, Y., SAITO, T., MURAMATSU, M. (1981)
Closely related mRNA sequences of Protamines in Rainbow
25   Trout Testis. J. Biochem. 89:1863-1868.

AYRES, H.M., PAYNE, D.N., FURR, J.R., RUSSELL, A.D. (1998)
EFFECT OF PERMEABILIZING AGENTS ON ANTIBACTERIAL ACTIVITY AGAINST A
SIMPLE PSEUDOMONAS AERUGINOSA BIOFILM. LETT. APP. MICROBIOL. 27: 79-
30   82

AIKEN,J.M., McKENZEI, D., ZHAO, H.-Z., STATES, J.C., DIXON, G.H. (1983)
Sequence homologies in the protamine gene family of rainbow trout. Nucleic Acids Res.11:4907-4922.

5

WORLD WIDE WEB (WWW) 2001
HTTP://WWW.DSMZ.DE/MEDIA/MED381.HTM WORLD WIDE WEB (WWW) 2001
http://www.dsmz.de/media/med381.htm

10   DREWS, G. (1983)
MIKROBIOLOGISCHES PRAKTIKUM, SPRINGER HEIDELBERG, NEW YORK, TOKIO.

PCT

0-1	Form - PCT/RO/134 (EASY) Indications Relating to Deposited Microorganism(s) or Other Biological Material (PCT Rule 13bis)	
0-1-1	Prepared using	PCT-EASY Version 2.92 (updated 01.01.2002)
0-2	International Application No.	
0-3	Applicant's or agent's file reference	1027

1	The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:	
1-1	page	72
1-2	line	2
1-3	Identification of Deposit	
1-3-1	Name of depositary institution	DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
1-3-2	Address of depositary institution	Mascheroder Weg 1b, D-38124 Braunschweig, Germany
1-3-3	Date of deposit	10 June 2002 (10.06.2002)
1-3-4	Accession Number	DSMZ 15050
1-4	Additional Indications	NONE
1-5	Designated States for Which Indications are Made	all designated States
1-6	Separate Furnishing of Indications These indications will be submitted to the International Bureau later	NONE
2	The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:	
2-1	page	72
2-2	line	3
2-3	Identification of Deposit	
2-3-1	Name of depositary institution	DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
2-3-2	Address of depositary institution	Mascheroder Weg 1b, D-38124 Braunschweig, Germany
2-3-3	Date of deposit	10 June 2002 (10.06.2002)
2-3-4	Accession Number	DSMZ 15051
2-4	Additional Indications	NONE
2-5	Designated States for Which Indications are Made	all designated States
2-6	Separate Furnishing of Indications These indications will be submitted to the International Bureau later	NONE

PCT

Original (für EINREICHUNG) - gedruckt am 14.06.2002 05:36:19 PM

2-6	Gesondert eingereichte Angaben Diese Angaben werden dem Internationalen Büro später übermittelt	KEINE
3	Die nachstehenden Angaben betreffen den Mikroorganismus und/oder anderes biologisches Material, der/das in der Beschreibung genannt ist	
3-1	Seite	72
3-2	Zeile	4
3-3	Angaben betr. Hinterlegung	
3-3-1	Name der Hinterlegungsstelle	DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
3-3-2	Anschrift der Hinterlegungsstelle	Mascheroder Weg 1b, D-38124 Braunschweig, Germany
3-3-3	Datum der Hinterlegung	12 Juni 2002 (12.06.2002)
3-3-4	Eingangsnummer	DSMZ 15052
3-4	Weitere Angaben	KEINE
3-5	Bestimmungsstaaten, für die besondere Angaben gemacht werden	alle Bestimmungsstaaten
3-6	Gesondert eingereichte Angaben Diese Angaben werden dem Internationalen Büro später übermittelt	KEINE

VOM ANMELDEAMT AUSZUFÜLLEN

0-4	Dieses Formular ist mit der Internationalen Anmeldung eingegangen (ja oder nein)	
0-4-1	Bevollmächtigter Bediensteter	

VOM INTERNATIONALEN BÜRO AUSZUFÜLLEN

0-5	Dieses Formular ist an folgendem Datum beim internationalen Büro eingegangen	
0-5-1	Bevollmächtigter Bediensteter	